MUTATIONS IN THE BRCA1 GENE

FIELD OF THE INVENTION

This invention relates to the cancer suppressor gene BRCA1. More specifically, this invention detects germline mutations of the BRCA1 gene that are associated with breast and ovarian cancer, and somatic cell mutations of the BRCA1 gene indicating the nature of the cancer. Methods and reagents for detecting the presence of these mutations are included.

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BACKGROUND OF THE INVENTION

BRCA1 is a putative tumor suppressor gene located on chromosome 17. Mutations in the BRCA1 gene are thought to account for roughly 45% of inherited breast cancer and 80-90% of families with increased risk of early onset breast and ovarian cancer (Easton, 1993, *et al.*, American Journal of Human Genetics 52: 678-701). A compilation of the known BRCA1 mutations may be found at the Breast Cancer Information Core world wide web site at http://www.nhgri.nih.gov/Intramural_research/Lab_transfer/Bic/ (BIC) (Friend, S. *et al.*, 1995, Nature Genetics 11: 238). The BRCA1 gene is approximately 100,000 base pairs of genomic DNA encoding the 1836 amino acid BRCA1 protein. The sequence is divided into 24 separate exons. Exons 1 and 4 are noncoding, in that they are not part of the final functional BRCA1 protein product. Each exon consists of 200-400 bp, except for exon 11 which contains about 3600 bp (Weber, B., Science & Medicine (1996). A consensus sequence for the coding region of the human BRCA1 gene, referred to herein as BRCA1(omi1), was first disclosed in Application No. 08/598,591 (as SEQ ID NO:1, therein), herein incorporated by reference.

Accuracy in detecting mutations in BRCA1 is extremely important, particularly in clinical settings. Direct end-to-end sequencing of the gene potentially provides the most reliable results, given that an accurate reference sequence is available. However, direct sequencing is a cumbersome technique. Detection of one of many known or unknown mutations is further complicated when the gene is large and/or has a complex structure. The human BRCA1 gene, for example, is approximately 100,000 base pairs long and contains 24 exons (Weber, B., Science and Medicine, Scientific American Jan-Feb. 1996, 12-21). Furthermore, in order to be practical and available to the general population, mutation detection methods must be efficient enough to accommodate a large number of different samples.

A number of techniques that are more rapid but less comprehensive than direct sequencing have been developed for detecting nucleotide sequence variations. These techniques may be used to detect differences between normal and mutant nucleotide sequences. DNA sequence-based allelic discrimination methods include: (1) allele-specific hybridization techniques, which effect detection under high stringent hybridization conditions; (2) single strand conformation polymorphism analysis and heteroduplex analysis which exploit differences in secondary structure of nucleic acid molecules; (3) denaturing gradient gel electrophoresis and constant denaturing gel electrophoresis which detect different alleles based on differences in melting behavior of nucleic acid molecules; (4) restriction enzyme cleavage, which discriminates between alleles based on the presence of absence of corresponding restriction recognition sequences; and (5) chemical or nuclease cleavage which detect base mismatch loci. Other techniques, such as the protein truncation test (Hogervorst F.B., et al., Nat. Genet. 1995 Jun;10(2):208-212), detect changes in the protein transcripts. For a summary of such techniques, see Marajver & Petty, 1996, Clinics in Lab. Med. 16: 139-167, especially Table 5 at p. 152.

One limitation of all these techniques is that sequence variations are often of unknown clinical significance. Since the triplet code is degenerate, many genetic variants do not alter the amino acid sequence of the resultant protein. Even those sequence alterations that do result in

amino acid changes may not have a significant impact on protein function. For example, some amino acid changes will substitute functionally similar amino acids (conservative substitutions) such as one small neutrally charged amino acid for another. Further, some regions of a protein molecule may not be important for protein function. In those regions of the molecule, even large changes the amino acid sequence may be possible. Thus, genetic variants as a whole are often of unknown clinical significance.

If an altered sequence in the coding region of a gene associated with a condition such as cancer is found however, it is important to determine the clinical significance of the variant sequence. Knowing the significance of the sequence variation can be an invaluable tool in determining an appropriate treatment or monitoring regimen. For example, if an individual carries a mutation that interferes with protein function, it may be possible to provide the individual with increased expression of the gene through gene transfer therapy. It has been demonstrated that the gene transfer of the BRCA1 coding sequence into human cancer cells inhibits their growth and reduces tumorigenesis in nude mice. The BRCA1 protein product appears to be a secreted tumor growth inhibitor, making BRCA1 an ideal gene for gene therapy studies. Transduction of only a moderate percentage of tumor cells apparently produces enough growth inhibitor to inhibit all tumor cells. Arteaga, C.L., and J.T. Holt Cancer Research 56: 1098-1103 (1996); Holt, J.T. et al., Nature Genetics 12: 298-302 (1996). The observation of Holt et al. that the BRCA1 growth inhibitor is a secreted protein, also suggests the possibility of tumor suppression by injecting the growth inhibitor into the area of the tumor.

Efforts have independently focused on increasing the efficiency of DNA sequence analyses and increasing the comprehensiveness of the sequence-based techniques. There remains a need, however, for a comprehensive method of detecting mutations in individual gene samples that is both accurate enough to provide a reliable diagnosis to an individual patient and efficient enough to be practical for application to the general population.

Until now, the art has relied upon the occasional occurrence of a previously unreported mutation to increase its base of information for mutation testing in a given gene sequence. To determine the presence or absence of a mutation in a gene from a patient sample, the vast majority of test samples would be subject to complete end-to-end sequencing of the gene. This method is time consuming, often taking six weeks to obtain a result, and is extremely expensive. To ascertain the clinical significance of a previously unidentified sequence variation, those in the art would frequently rely on epidemiological data derived from an analysis of families or populations carrying the newly identified mutation. Other methods of assessing the significance of a newly identified sequence variation would include cloning the altered gene and studying its function in vitro or in vivo or comparing the altered gene sequence with homologous genes in other organisms. Given the realities of many of these genetic diseases, such an analysis comes too late to be of use to the individual bearing the newly identified sequence variation.

There is need in the art, therefore, for improved methods to identify clinically significant mutations. Identification of mutations of the BRCA1 gene would allow better tumor cell analysis for more appropriate therapy, and more widespread diagnostic screening for hereditary cancers than is currently possible, and also permit identification of critical or biologically significant functional areas deduced from the mutational spectrum observed. While mutations occur throughout the BRCA1 gene, there is a need for an assay, test or means for detecting mutations with a high sample number (throughput), sensitivity, accuracy and cost effectiveness.

The present invention addresses these needs and more by providing mutations, molecules, and methods useful for the identification of both known and unknown mutations.

SUMMARY OF THE INVENTION

It is an object of the invention to provide a method for determining a predisposition or higher susceptibility to cancers in individuals by determining the DNA sequence of the BRCA1 genes in the DNA of a patient specimen and comparing it to a naturally occurring (wild type)

BRCA1 gene sequence or any haplotype thereof resulting from polymorphisms to determine whether the sample contains a mutation.

It is a further object of the invention to provide a method of characterizing and classifying a tumor and determining an appropriate therapy dependant upon the type of BRCA1 mutation(s) present.

It is also an object of the present invention to provide a non-chromosomal mutant BRCA1 gene and expressed mutant protein for drug development, gene therapy and other uses to prevent or ameliorate the effects of or resulting from the mutant BRCA1 gene.

It is another object of the present invention to prepare oligonucleotides, or groups of these oligonucleotides, where the oligonucleotide will specifically hybridize to either the wild type BRCA1 gene or a mutant BRCA1 gene to distinguish between the two BRCA1 genes.

It is still another object of the present invention to assay for the presence of a BRCA1 gene encoding a truncated BRCA1 protein by testing for the presence of a truncated BRCA1 protein.

The present invention is based on the discovery of numerous mutations in the published BRCA1 DNA sequence that result in a truncation of the BRCA1 protein. Mutations in the BRCA1 gene that cause truncations are associated with increased susceptibility to, and developmental stage of, different cancers, particularly breast and ovarian cancer. A truncated protein is likely to be non-functional or at least have a different biological activity.

DETAILED DESCRIPTION OF THE INVENTION

The present invention stems, in part, from the realization that previously unidentified sequence variations as a whole are typically of unknown clinical significance. At this time, epidemiological studies are time-consuming and may be of little use for infrequently identified sequence variations; laboratory studies of protein function are similarly time-consuming and difficult to extrapolate to human disease. However, mutations resulting in a prematurely

terminated protein have a high probability of having clinical significance. In part, this is due to the fact that a premature termination of a coding sequence may entirely delete important functional domains, such as zinc fingers, transmembrane domains, phosphorylation sites, glycosylation sites, etc. It is also due, in part, to the fact that the removal of a portion of a protein molecule can significantly disrupt a protein's structure and function, even where no obvious functional domain has been deleted. Thus, the larger the truncation, the greater the likelihood that an important part of the molecule has been removed. Regardless of the cause, a survey of known clinically significant sequence variations (mutations) of the BRCA1 gene reveals that a large percentage of identified mutations are premature truncation mutations of the BRCA1 protein product. Thus, identifying those sequence variations that are premature truncation-causing mutations offers enormous predictive value to the clinician.

The presence of tumor cells with a mutation that inactivates BRCA1 may be clinically significant in determining the tumor stage, likelihood of metastasis, appropriate therapy etc. BRCA1 mutations appear in advanced tumors, several advanced primary cancers and cell lines derived from highly aggressive cancers. Individuals with one of their alleles having a BRCA1 mutation in their germline are at increased susceptibility for cancer, particularly breast and ovarian cancer.

With DNA sequencing technology, genomic DNA has been extracted from samples of whole blood, a cell line or a tumor and the coding regions of the BRCA1 gene were amplified using the polymerase chain reaction (PCR). Each of the coding regions has been sequenced completely and is recited in GENBANK, Accession Number I59546.

A number of mutations have been described in the BRCA1 gene that cause stop codons to be formed, thereby encoding a truncated BRCA1 protein. A list of mutations published to date is in TABLE 1.

The nomenclature of the published literature is inconsistent. See, for example, Beaudet *et al*, Human Mutations, 2: 245-248 (1993), Antonarakis *et al*, Human Mutations, 4: 166 (1994),

Cotton, Human Mutations, 8: 197-202 (1996), and Beutler *et al.*, Human Mutations, 8: 203-206 (1996). Consequently, the following nomenclature is used to define the mutations and polymorphisms of the present invention. In defining the mutation, the number indicates the nucleotide number corresponding to the BRCA1 gene sequence where the mutation first occurs. For simplified identification purposes, the BRCA1 sequence of SEQ ID NO:1, of US Patent 5654155 (GENBANK Accession number I59546) is used. However, the invention is equally applicable to other BRCA1 sequences. Other BRCA1 sequences (haplotypes) that are polymorphisms or genetic variations of BRCA1 may be used, in which case, a corresponding mutation at the corresponding nucleotide number is present.

Haplotypes are distinguished based on the combination of nucleotides present at particular polymorphic loci. Polymorphic nucleotide sites 2201, 2430, 2731, 3232, 3667, 4427, and 4956 usually define a haplotype. Other polymorphic sites of a BRCA1 gene are at Exon 4 (not coding) 49, IVS8-57, 1186, 2196, and 3238. Less common polymorphisms are at 233G>A, 561-34C>T, exon 9-6delT, 710C>T, 1100A>G, 1985G>T, 2202G>A, 2687T>C, 2933A>G, 3263T>C, 4077T>C, 4145A>G, 4193G>A, 4209-141C>A, 4364A>G, 4932T>C, 5106-68G>A, 5106-92G>A, intronic exon17-G>A, 5232G>T, 5272+66A>G, intronic exon18+A>G, 5396+48 12bp insert, 5396+47 12bp insert, intronic exon 22+T>C, 5651C>T, 5657G>A, and UGA+36C>G. See, Couch *et al.*, Human Mutations, 8(1):8-18 (1996), where polymorphisms have been reported.

As the BRCA1 gene numbering does not include introns, when the mutation is located within an intron, a nucleotide in the coding sequence + or - a number of nucleotides is given. Insertion mutations are indicated by "ins" and deletion mutations are indicated by "del". Letters after "ins" or "del" refer to the nucleotide(s) which were inserted or deleted. Alternatively, where several bases have been inserted or deleted, a number may follow "ins" or "del," indicating the number of affected bases. When the mutation results in one nucleotide being substituted for another, the original nucleotide(s) is placed to the left of the nucleotide number and the

nucleotide(s) corresponding to the substituting nucleotide is placed to the right of the nucleotide number. In some cases, the substitution is indicated by both the original and the substituted nucleotides following the nucleotide number, with an ">" between them. In such cases the original nucleotide is to the left of the ">" and the substituted nucleotide is to the right of the ">," as for example, in the designation 5657G>A.

TABLE 1

185delAG, 185insA, 188del11, 189insA, 189insTGTC, 192del2, 259insT, 351delA, 448insA, 448delAG, 525insA, 589delCT, 613insT, 633delC, 787insA, 788delA, 794delT, 795delT, 816delGT, 916delTT, 917delTT, 926ins11, 962del4, 1048delA, 1049delG, 1099delCA, 1100delAT, 1103insC, 1129delA, 1135insA, 1191delC, 1201del11, 1205delGA, 1206delA, 1220insC, 1240delC, 1294del40, 1323delG, 1374delG, 138del29, 1395delT, 1406insA, 1411insT, 1438delT, 1459insG, 1479delAG, 1499insA, 1505delG, 1506delA, 1509delA, 1511insC, 151insC, 1559insA, 1611delC, 1623del5, 1670delT, 1675delA, 1701del7, 1768delA, 1832del5, 1942del4, 1996ins4, 2000del4, 2012insT, 2071insA, 2072del4, 2072insG, 2080delA, 2080delA, 2080insA, 2138delA, 2140delC, 2187delA, 2190delA, 2198delCA, 2229delAA, 2274insA, 2294delG, 2295delC, 2307insG, 2312del5, 2313del5, 2314del5, 231delAA, 2325delG, 2329delC, 2329delCA, 2334insCT, 2388delG, 2401delAA, 2415delAG, 2448delT, 2473insA, 2509delAA, 2569delG, 2575delC, 2576delC, 2594delC, 2594insA, 2595delA, 2596delC, 2711delA, 2731insT, 2765delTGC, 2795del4, 2798del4, 2800delAA, 2804delAA, 2809insA, 2819delTT, 2844del4, 2846delTCAA, 2862delTC, 2867insA, 2883del4, 2911delTGGT, 2925del4, 2953delGTA>insC, 2953insCdelGTA, 2954insT, 2982del5, 3039delTT, 3109insAA, 3121delA, 3124delA, 3135del4, 3166ins5, 3172ins5, 3345delAG, 3345delAG, 3375insGA, 339insA, 3407delAA, 3411delCT, 3449insA, 3450del4, 3476delT, 3596del4, 3599del11, 3600del11, 3604delA, 3668delAGinsT, 3731delA, 3768insA, 3818delA, 3819del5, 3825del8, 3829delT, 3874del4, 3875del4, 3879insT, 3879insT, 3883insA, 3889delAG, 3890delGG, 3896delT, 3938insG, 3960delCA, 3977del4, 3988delAA, 4035delTT, 4050del4, 4091delG, 4154delA, 4184del4, 4239delAG, 4280delTC, 4284delAG, 4601delAA, 4693delAA, 5055delG, 5061delA, 5083del19, 5085del19, 5085del19, 5124delG, 5124delG, 5145del11, 5145del11, 5149del4, 5149del4, 5154del5, 5154del5, 5245delG, 5256delG, 5296del4, 5348delAA, 5382insC, 5389del7, 5404insG, 5438insC, 5439delAA, 5502insT, 5559delG, 5598insGA, 5629delG, 5640delA, 5677insA, A1518T, A2154T, C1599T, C1648G, C1695T, C1740T, C1806T, C2257G, C2428A, C2457T, C297T, C3042T, C3508G, C3549T, C3726T, C3726T, C3726T, C3726T, C3837T, C3904A, C3960T, C3960T, C3960T, C4086T, C4302T, C4305T, C4341T, C4377T, C4446T, C4808G, C4929T, C5370T, C5622T, C624T, G1081A, G1177A, G1235A, G1371T, G1569T, G2307T, G2508T, G2841T, G3297T, G3759T, G3780T, G3867T, G4740T, G5199T, G5273A, G5292T, G5465A, G546T, G5563A, G5625T,

G5630A, IVS12-1643del3835, IVS12-1643del3835, T1411G, T2035A, T3053G, T3358A, T3376G, T3458G, T5298A.

It should be noted that polymorphisms in the coding sequence are known and listed in TABE 2. An example is A180G. In the non-coding regions, a deleted T, 2 base pairs beyond exon 19 (5312+2delT) and a 12 base pair insertion 47 bases beyond exon 20 (5396+47ins12bp) are also known polymorphisms. Other genetic changes which may be polymorphisms or mutations are listed under mutations above. While the present invention references the sequences recited above, it is recognized that polymorphisms, either these recited or others, may be equally used for the purposes of the present invention.

TABLE 2

del exon 3, del561-702, del561-789, exon 18 delA, 5312+2delT, codong 1749 Pro to Arg, IVS20ins12, dup5396 +48, A1100G, G1112C, C1120T, A1186G, A1186G, A120G, M1I, D369del, T1256G, G1503A, T151C, A1546G, T1575C, C1605T, G1606A, G1630A, G1639T, T172C, C1735T, A1767C, A180G, 5544delGTT, C1822T, T1831C, T189C, G1985T, exon 9 - 2A>C, C2053A, G2093C, C212G, C2121T, G2196A, C2198T, C2201T, G2202A, A2286G, C2299T, G233A, G233A, A243G, T2430C, T2434C, G2531C, A2577G, C2596A, C2596A, C2640T, A2646G, T2687C, C2715T, C2731T, C2731T, C2731T, A2933G, T300G, C3030A, T309G, G310A, G310A, G3143A, A3165G, G3202A, A3232G, A3233G, G3238A, G3238A, T3263C, A330G, In6(+3)A>G, A3339G, C3415T, A3446G, T3529C, A3537G, G3543C, C3547T, C3567T, C3567T, A3667G, C3706A, G3720A, G3727A, G3776C, T378G, C3832T, G3867A, T388C, T4077C, A4145G, G4155A, A4158G, G4193A, 4209-141C>A, G4303A, A433G, G4364A, C4380T, C4427T, C4446G, Exon12+6,T>C, G4603T, G4654T, G4719A, G4755A, G5396+48ins12bp, C4801T, C4801T, IVS7-3delT, C49T, A4931G, T4932C, A4935G, C4942T, A4956T, A4956G, A5001G, T5002C, T5002C, C5029T, G5040A, G5075A, G5076A, G5112A, G5112A, V1688del, V1688del, G5193A, G5193A, 5194-2A>C, C5214T, G5215A, C5232T, C5242A, C5242A, T5257C, G5263A, IVS18+1G>T, 5272+66A>G, I-18 5272+66G>A, 5272+66G>A, A5277G, 5280delCAG, A5317G, G5332A, A5335G, G5396A, 5396+1 G>A, G5396+1A, 5396+47ins12bp, T5443G, G546A, T5467C, G5482T, T5530A, C5535G, T5542C, T5548G, A5575G, G5586A, 561-34 C>T, G5616A, T5628C, C5651T, 5657G>A, A655G, C676A, G690A, C710T, C710T, G731C, T855G, G876A, G930A, G930C, exon 2 -3insAG , IVS20+60ins12, Intron22T>C, T>G ins59 bp, exon 9 -6delT, G5396+47ins12bp, in18+1G>C, in20-1(G>T), IVS11-2delGT, IVS20ins12, IVS22+5G.A, IVS1-21insAT, IVS11-2A>G, IVS13+1G>T, IVS13+2T>G, IVS13-10C>T, IVS14-2A>G,

IVS15+1G>A, IVS16+3G>C, IVS16+6T>C, IVS16-20A>G, IVS16-20A>G, IVS18+6T>G, IVS18-13A>G, IVS2+1G>A, IVS2+1G>T, IVS2-11delT, IVS2-12C>G, IVS20+60ins12, IVS20-1G>A, IVS22+8T>C, IVS23-10C>A, IVS4-1G>T, IVS5+1G>T, IVS5-11T>G, IVS5-12A>G, IVS6+7G>A, IVS6-2A>T, IVS6-2delA, IVS7-3delT, IVS8+2T>A, IVS8-17G>T, IVS9+3G>A, exon24(+36)C>G, 3'UTR C>G (+36).

It should be noted that not all of the mutations listed in TABLES 1 and 2 are prior art as some of the mutations were published very recently after applicant determined his mutations.

Mutations detected according to the present invention are nonsense and frame shift mutations. Nonsense mutations cause an in-frame stop codon, which results in expression of a truncated protein of presumably no BRCA1 functional ability or at least a significantly altered BRCA1 biological activity. Frameshift mutations cause an out-of-frame stop codon to be created in the inserted or deleted coding sequence. This formed stop codon may be at the site of mutation or downstream from the mutation. For the example of nonsense mutations, a nucleotide in a codon is mutated to provide a stop codon having a sequence of TAA, TAG or TGA. For the example of a frame shift mutation, 1, 2, 4, 5, or any larger integer not 3 or a multiple of three, nucleotides are inserted into, or deleted from, the BRCA1 gene sequence. Such mutations result in the formation of a stop codon at the codon containing the mutation or within codons downstream from the mutation site, but before the end of the wild type BRCA1 gene.

The presence of one or more of such mutations is expected to be clinically significant as they are capable of producing a truncating BRCA1 mutation. As the effects of truncations are predictably harmful, one has a high degree of certainty that the presence of such a mutation is clinically significant.

For example, truncating mutations 5382insC, 5438insC, and 185delAG are known to be associated with cancer (Abeliovich, *et al.*, Am. J. Hum. Genet., 60:505-514 (1997); Struewing *et al.*, Nat. Genetics, 11:198-200 (1995); Shattuck-Eidens, *et al.*, JAMA 273(7):535-541 (1995). Accordingly, any truncating mutation deleting the same portion or more of the coding sequence

is presumed to produce an affected mutant BRCA1 protein and to be associated with cancer or an increased susceptibility to cancer. 5382insC and 5438insC cause frame shifts, producing a stop codon (TGA) at nucleotides 5604-5606. 185delAG causes a frame shift, producing a stop codon (TGA) at nucleotides 234-236. Therefore, for the purposes of the present invention, truncating mutations at this location of a lower nucleotide number are presumed to be harmful or of clinical significance.

Conversely, the clinical significance of many missense mutations is unclear. Even when isolated from a tumor cell, the exact effect on BRCA1 protein must be independently shown. Theoretically, most variations in the BRCA1 sequence will be missense variations and relatively fewer will be truncating mutations. At the present time, the rules are unknown for which missense mutations in BRCA1 are predictably harmful or otherwise clinically significant. The BRCA1 gene is simply not sufficiently characterized, and given the history of mutations in other genes and computer programs attempting to find predictability, missense mutations will continue to be unpredictable. Without a certainty of their clinical significance, simply knowing that a missense variation exists may have no value.

In general, truncated proteins are non-functional or of reduced function by lacking proper biological activity. The presence of a BRCA1 gene encoding a truncated protein has a high probably of being clinically significant. This is in part due to important portions of the BRCA1 protein being located in the truncated region and not present in the expressed mutant protein. Important functional domains include, for example, any part of the active site, transmembrane domains, zinc fingers, phosphorylation sites, glycosylation sites, sites interacting with metal ions, coenzymes, cofactors, other ligands or receptors, etc. Furthermore, removal of part of the protein molecule or addition of amino acids to the sequence can significantly disrupt the secondary, tertiary or quaternary (if any) structure of the protein, even when no obvious functional domain has been deleted by the truncation.

For example, the 5382insC mutation is found in breast and ovarian cancers. This single base insertion creates a stop-codon at codon 1829, truncating the protein at a cysteine residue. This truncation deletes less than 2 percent of the coding sequence of BRCA1. Accordingly, all truncations that delete this region of the BRCA1 gene would also by inference render the truncated BRCA1 protein non-functional for its putative tumor suppressor function. Mutant BRCA1 genes with even smaller portions of the gene being truncated are also known to be associated with cancer. For example, truncating mutations causing a stop codon at nucleotides 5676-5678, truncating 0.5% of the coding sequence, are also known to be associated with breast cancer (5677insA; Shattuck-Eidens *et al.*, JAMA, 273(7):535-541 (1995)). Please note that several mutations in the DNA result in the expression of the same mutant BRCA1 protein because each mutation causes a stop codon to be formed at nucleotide site 5676-5678. This corresponds to codon 1853 (TAC - Tyrosine). Thus, all of these mutations are very likely to cause the same effective result, for example, in terms of susceptibility to cancer or cancer typing. Therefore, detecting these mutations would be diagnostically significant and useful for assessing the susceptibility for cancer. Given these mutations, oligonucleotides complementary to the regions of these mutations are prepared to assay for the presence of these mutations in a sample.

For the present invention, the truncating mutations are detected in the BRCA1 gene or a fragment thereof which contains a mutation which directly or indirectly causes the formation of an in-frame stop codon (TAA, TAG or TGA) prematurely at any of codons 2 to 1863 A premature in-frame stop codon is one that occurs before the natural stop codon, which does not occur in a wild-type BRCA1 gene and when expressed results in a truncated protein product.

These BRCA1 mutations of the present invention may also be defined as specifically hybridizing to an oligonucleotide probe having the sequence 5' R1-R2-R3 3' wherein R1 is an oligonucleotide of at least three nucleotides, R2 is complementary to TAA, TAG or TGA, and R3 is an oligonucleotide of at least three nucleotides, the probe hybridizing to a premature in-frame stop codon on the mutant BRCA1 gene.

Whenever defining an oligonucleotide probe in the present invention, probes capable of potentially hybridizing to the sense strand are referred to. It should be recognized that oligonucleotide probes having a sequence complementary to these oligonucleotide probes may also be used and may specifically hybridize to the anti-sense strand. For diagnostic purposes either may be used, as DNA from biological samples is generally double stranded and contains both the sense and anti-sense strands.

In the present invention, the following BRCA1 mutations of the present invention represent one base changes that result in the formation of an in frame TAA, TAG or TGA. These mutations of the present invention are defined by forming stop codons at specific locations in accordance with TABLE 3. Any expressed protein from BRCA1 genes with these types of mutations should be truncated accordingly. The substituted nucleotide is indicated in lower case letters.

TABLE 3
List of Nonsense Mutations

Stop Codon Formed	Nucleotide Number	Base Change
TaA	127	T>A
TgA	127	T>g
tAA	144	G>T
tAA	147	G>T
tAA	153	C>T
tAG	174	C>T
tAA	177	A>T
TaA	184	T>A
TgA	184	T>g
tAG	186	G>T
TGa	191	T>A
TGa	200	T>A
tAG	204	G>T
TaG	208	T>A
tAG	213	A>T
tAA	216	G>T

tAG	231	A>T
TGa	236	T>A
TGa	251	C>A
tAA	252	A>T
TGa	260	C>A
tAA	267	A>T
tAG	279	C>T
tAG	282	A>T
tAA	285	A>T
TaA	295	C>A
TgA	295	C>g
tAG	297	C>T
TGa	302	T>A
TaA	307	T>A
TgA	307	T>g
TGa	311	T>A
tAG	312	A>T
tAA	327	A>T
tAA	339	C>T
tAA	342	G>T
tGA	351	A>T
tAA	360	C>T
tAA	369	G>T
tAG	372	G>T
TaG	379	T>A
tAA	381	A>T
TGa	392	T>A
tAG	399	C>T
TaG	415	T>A
tAG	417	G>T
TAa	422	T>A
TAg	422	T>G
TAa	434	T>A
TAg	434	T>G
tAA	444	A>T
tAG	447	A>T
tAA	450	G>T
tAA	465	G>T
tAA	474	A>T

tAA	480	G>T
tAA	495	C>T
TAa	509	C>A
TAg	509	C>G
tGA	510	A>T
tAA	522	A>T
tGA	525	A>T
tAG	534	C>T
tAA	540	G>T
tAA	546	G>T
TaG	559	T>A
tAG	561	C>T
tAA	564	G>T
tAA	582	C>T
tGA	597	G>T
tGA	606	A>T
tAG	621	A>T
tAG	624	C>T
tAA	633	C>T
tAA	639	C>T
tAG	642	A>T
TAa	656	C>A
TAg	656	C>G
tAA	660	G>T
TaG	664	T>A
tGA	666	G>T
tAA	681	G>T
tAG	696	A>T
TAa	707	T>A
TAg	707	T>G
TGa	710	C>A
tGA	717	G>T
tAA	723	C>T
tAA	726	G>T
TaG	730	T>A
TaA	733	T>A
TgA	733	T>g
tAA	735	C>T
tAA	747	C>T

tGA	750	G>T
tAA	762	G>T
TaG	772	T>A
tAA	783	A>T
tAG	786	A>T
TGa	797	T>A
tAA	798	G>T
tAG	807	G>T
tAA	828	G>T
tAA	837	C>T
TaG	856	T>A
tAG	867	G>T
tAG	870	A>T
tAG	882	G>T
tAA	894	G>T
tAG	897	A>T
TAa	902	T>A
TAg	902	T>G
tAG	903	C>T
TaA	919	C>A
TgA	919	C>g
TaG	925	T>A
tAG	933	G>T
TGa	941	T>A
TaA	964	C>A
TgA	964	C>g
TaA	967	T>A
TgA	967	T>g
tAG	969	C>T
tAG	975	G>T
TaA	988	T>A
TgA	988	T>g
TaA	991	T>A
TgA	991	T>g
tAA	999	A>T
tGA	1005	A>T
tAA	1017	G>T
tAG	1020	A>T
tAA	1026	G>T

TGa	1034	T>A
tAA	1038	A>T
tAA	1044	A>T
tAG	1047	C>T
TaA	1057	T>A
TgA	1057	T>g
tAA	1068	C>T
tGA	1077	A>T
TaG	1081	G>A
TGa	1082	G>A
tGA	1086	G>T
tAG	1092	A>T
tAA	1095	G>T
TGa	1103	T>A
tAA	1128	G>T
tAA	1131	A>T
tAG	1134	A>T
TGa	1163	T>A
tAG	1164	G>T
tGA	1167	A>T
tAA	1170	A>T
tAA	1173	G>T
TaG	1177	G>A
TGa	1178	G>A
tAG	1182	A>T
tAG	1185	C>T
tAA	1188	A>T
TGa	1199	C>A
TaA	1201	C>A
TgA	1201	C>g
tAG	1203	G>T
tGA	1212	A>T
tAA	1221	G>T
TaG	1234	G>A
TGa	1235	G>A
tAG	1257	C>T
tAA	1260	A>T
tAG	1269	G>T
TaG	1273	G>A

TGa	1274	G>A
tGA	1281	A>T
tAA	1290	G>T
TaA	1297	T>A
TgA	1297	T>g
TaA	1312	C>A
TgA	1312	C>g
tAG	1323	G>T
tAA	1329	G>T
TaA	1333	C>A
TgA	1333	C>g
tAA	1341	A>T
TaG	1357	T>A
tAG	1371	G>T
tAA	1380	G>T
TAa	1385	T>A
TAg	1385	T>G
TaA	1396	C>A
TgA	1396	C>g
tAG	1398	G>T
tAA	1401	A>T
TaA	1411	T>A
TgA	1411	T>g
tAG	1431	G>T
TaA	1438	T>A
TgA	1438	T>g
TGa	1445	T>A
tAA	1446	A>T
tAA	1452	G>T
tGA	1455	A>T
tAA	1467	A>T
TaA	1471	C>A
TgA	1471	C>g
tAG	1476	G>T
tAA	1488	G>T
tAA	1494	A>T
tAA	1506	A>T
TAa	1514	T>A
TAg	1514	T>G

tAG	1518	A>T
tAG	1521	A>T
TaA	1540	T>A
TgA	1540	T>g
tAA	1554	G>T
tGA	1569	G>T
tAG	1584	G>T
tAG	1590	C>T
tAA	1599	C>T
tAG	1602	G>T
tAA	1620	A>T
TaA	1624	T>A
TgA	1624	T>g
tAG	1626	A>T
tAA	1632	A>T
tGA	1638	A>T
TaA	1648	C>A
TgA	1648	C>g
tAG	1662	G>T
tAG	1674	A>T
tAA	1677	A>T
TaG	1687	T>A
tAA	1695	C>T
tAG	1698	A>T
tAA	1707	G>T
tAG	1719	C>T
tGA	1722	G>T
tAA	1731	C>T
tAG	1737	G>T
tAG	1740	C>T
tAA	1749	C>T
tAG	1779	G>T
tAA	1785	A>T
tAA	1791	A>T
tAG	1806	C>T
tAG	1812	G>T
tAA	1815	A>T
tAA	1833	G>T
TaA	1837	C>A

TgA	1837	C>g
tAA	1842	G>T
tAA	1845	A>T
tAA	1848	G>T
tAA	1860	A>T
tAA	1866	A>T
tAA	1872	G>T
tAA	1902	G>T
tAA	1908	G>T
TaA	1912	T>A
TgA	1912	T>g
TaA	1927	C>A
TgA	1927	C>g
tAA	1929	A>T
tAA	1938	A>T
tAG	1941	A>T
tAG	1959	A>T
tAA	1989	G>T
tGA	2004	A>T
TGa	2027	T>A
tAA	2031	G>T
TaG	2035	T>A
tAA	2037	C>T
TGa	2051	T>A
tAA	2061	G>T
tAG	2064	G>T
tAG	2070	A>T
tAA	2073	A>T
tAA	2076	A>T
tAG	2079	A>T
TAa	2084	C>A
TAg	2084	C>G
tAA	2088	C>T
tGA	2109	A>T
tAA	2118	C>T
tAA	2127	G>T
tAA	2133	A>T
tAA	2136	G>T
tGA	2148	G>T

tAG	2154	A>T
tAG	2157	A>T
tAG	2166	A>T
tAA	2175	G>T
tAG	2178	C>T
tAA	2187	A>T
tGA	2190	A>T
tAG	2214	G>T
tAG	2220	A>T
TaA	2224	T>A
TgA	2224	T>g
tAG	2250	A>T
TGa	2255	T>A
TaA	2257	C>A
TgA	2257	C>g
tAA	2268	G>T
tAA	2274	A>T
tAA	2277	G>T
tGA	2301	A>T
tAA	2304	G>T
tAA	2307	G>T
tAA	2310	A>T
tAA	2313	G>T
tAG	2316	G>T
tAA	2319	A>T
tAA	2325	G>T
tAA	2334	A>T
tAA	2352	G>T
tAA	2361	A>T
TaA	2374	T>A
TgA	2374	T>g
tGA	2379	G>T
tAA	2382	G>T
TaG	2392	T>A
tAA	2394	C>T
tAA	2400	G>T
tGA	2403	A>T
tAG	2412	G>T
TaA	2428	C>A

TgA	2428	C>g
TAa	2450	T>A
TAg	2450	T>G
tAG	2457	C>T
tAA	2460	G>T
TaG	2470	C>A
TaA	2473	T>A
TgA	2473	T>g
tAA	2478	G>T
tAG	2496	A>T
tAA	2502	A>T
tAA	2508	G>T
tAA	2517	A>T
TGa	2522	T>A
tAG	2529	C>T
TGa	2534	T>A
tAA	2544	G>T
tAG	2553	A>T
tGA	2556	G>T
TGa	2573	T>A
tAA	2577	A>T
tGA	2586	A>T
tAA	2598	G>T
tAG	2607	A>T
TAa	2612	T>A
TAg	2612	T>G
TaG	2617	T>A
tGA	2619	G>T
tAA	2625	G>T
tAA	2643	G>T
tAA	2655	G>T
tAA	2661	G>T
tAA	2664	G>T
tAA	2670	G>T
tAG	2682	C>T
TAa	2687	T>A
TAg	2687	T>G
TaG	2689	T>A
tAG	2691	C>T

tAG	2703	A>T
TaA	2710	C>A
TgA	2710	C>g
tAG	2712	A>T
tAG	2718	C>T
TaA	2722	C>A
TgA	2722	C>g
TaA	2737	C>A
TgA	2737	C>g
tGA	2745	G>T
tAA	2754	G>T
tAG	2757	G>T
tAA	2760	G>T
TGa	2765	T>A
TaA	2794	T>A
TgA	2794	T>g
tAG	2796	A>T
tAA	2799	A>T
tAA	2802	C>T
tAA	2811	A>T
tAA	2823	G>T
TGa	2828	T>A
tAA	2829	G>T
tAA	2832	C>T
tAG	2835	A>T
tAA	2838	G>T
tAA	2841	G>T
tAA	2847	C>T
tGA	2850	G>T
tAG	2853	A>T
tAG	2859	G>T
tAG	2871	A>T
tAG	2880	C>T
tAG	2919	C>T
tAA	2922	A>T
tAG	2928	A>T
tAA	2946	A>T
TGa	2951	T>A
tAA	2958	A>T

tGA	2961	G>T
TGa	2978	T>A
TaA	2983	C>A
TgA	2983	C>g
tAG	2988	C>T
tGA	2994	A>T
tAA	3003	G>T
tGA	3009	G>T
tAA	3027	A>T
tGA	3033	G>T
TaA	3040	T>A
TgA	3040	T>g
tAA	3042	C>T
TAa	3053	T>A
TAg	3053	T>G
tAG	3078	A>T
TaA	3082	C>A
TgA	3082	C>g
tAA	3090	A>T
tAA	3096	A>T
TGa	3101	T>A
tAG	3102	A>T
tAA	3105	A>T
tAG	3117	G>T
tAA	3120	G>T
tAG	3129	G>T
tAA	3132	G>T
TaA	3139	C>A
TgA	3139	C>g
TaA	3145	C>A
TgA	3145	C>g
tAA	3150	G>T
tGA	3153	A>T
tAA	3156	G>T
tGA	3162	G>T
tAG	3168	G>T
tGA	3213	A>T
tAA	3216	G>T
tAA	3228	A>T

tGA	3231	G>T
TaA	3241	C>A
TgA	3241	C>g
tAA	3255	G>T
tAA	3276	G>T
tAA	3297	G>T
tAA	3315	G>T
tAA	3324	C>T
tAA	3330	G>T
tGA	3339	A>T
tGA	3345	A>T
tAA	3354	A>T
TaG	3358	T>A
tGA	3372	A>T
TaA	3376	T>A
TgA	3376	T>g
TaG	3385	T>A
tAA	3387	C>T
tAG	3393	G>T
TAa	3401	T>A
TAg	3401	T>G
tAA	3402	A>T
tAA	3405	C>T
tGA	3417	G>T
TGa	3428	T>A
tAG	3429	A>T
tAA	3438	G>T
tAA	3444	A>T
tAG	3447	A>T
tAA	3450	C>T
tAA	3453	G>T
TAa	3458	T>A
TAg	3458	T>G
tAA	3459	G>T
tAA	3462	G>T
tAG	3471	C>T
TAa	3500	T>A
TAg	3500	T>G
TaA	3508	C>A

TgA	3508	C>g
TaA	3517	T>A
TgA	3517	T>g
tAA	3519	G>T
tAG	3522	C>T
tGA	3531	G>T
tAG	3549	C>T
TGa	3557	T>A
tAG	3561	G>T
TaA	3580	T>A
TgA	3580	T>g
tAA	3591	G>T
tAG	3597	A>T
tAA	3600	G>T
tAA	3618	G>T
tAG	3630	A>T
tAA	3633	G>T
tAA	3654	A>T
tAG	3663	C>T
tGA	3666	A>T
tGA	3669	G>T
tAG	3672	G>T
TaG	3712	T>A
tAG	3717	C>T
TAa	3725	C>A
TAg	3725	C>G
tGA	3726	C>T
tGA	3729	A>T
tAG	3738	A>T
tAA	3741	A>T
TaA	3745	T>A
TgA	3745	T>g
tAG	3747	G>T
TaA	3754	C>A
TgA	3754	C>g
tAA	3756	G>T
tAG	3759	G>T
TaA	3766	T>A
TgA	3766	T>g

tAG	3774	G>T
tAA	3780	G>T
tAG	3783	G>T
TGa	3794	C>A
tAA	3798	C>T
TaG	3805	T>A
TaA	3808	T>A
TgA	3808	T>g
tAA	3816	A>T
tAG	3837	C>T
tAG	3867	G>T
TGa	3872	T>A
tAG	3879	A>T
tAG	3888	G>T
tAG	3891	G>T
TaA	3898	T>A
TgA	3898	T>g
TaA	3901	T>A
TgA	3901	T>g
TaA	3904	C>A
TgA	3904	C>g
TaG	3907	T>A
tAG	3909	A>T
TaA	3919	T>A
TgA	3919	T>g
TGa	3929	C>A
tAG	3936	C>T
TaG	3946	T>A
tAG	3951	A>T
tAG	3960	C>T
tAA	3963	G>T
tAG	3978	G>T
tAA	3981	G>T
tAA	3987	A>T
TGa	3992	T>A
TaG	4003	T>A
TaA	4012	C>A
TgA	4012	C>g
tAG	4014	C>T

TGa	4019	C>A
tAA	4023	G>T
TaG	4027	T>A
tAA	4029	G>T
TaG	4036	T>A
tAG	4056	C>T
TaG	4069	T>A
tAA	4083	A>T
tAA	4086	C>T
tAG	4098	C>T
tAA	4104	G>T
tAG	4110	C>T
tGA	4113	G>T
tAG	4131	A>T
tAA	4134	G>T
TaG	4138	T>A
TaA	4144	C>A
TgA	4144	C>g
tAA	4152	G>T
tAA	4155	G>T
tGA	4158	A>T
tGA	4161	G>T
TaG	4171	T>A
tAA	4173	G>T
tAA	4176	G>T
tAA	4185	C>T
tAA	4188	G>T
tAG	4191	G>T
tAA	4194	C>T
TaA	4207	C>A
TgA	4207	C>g
TaA	4213	T>A
TgA	4213	T>g
tAA	4218	G>T
TGa	4235	T>A
tAG	4236	G>T
tAA	4242	G>T
tAA	4257	G>T
TGa	4265	C>A

TaA	4267	C>A
TgA	4267	C>g
tAG	4281	C>T
TaA	4294	T>A
TgA	4294	T>g
tAG	4302	C>T
tAG	4305	C>T
tAA	4320	C>T
tAG	4335	A>T
tAG	4341	C>T
tAG	4344	C>T
tAA	4347	G>T
tAA	4356	G>T
tAA	4362	G>T
TaA	4372	T>A
TgA	4372	T>g
tAA	4374	G>T
tAG	4377	C>T
tAG	4389	C>T
TAa	4406	C>A
TAg	4406	C>G
tAG	4437	G>T
tGA	4446	C>T
tAA	4455	G>T
tAA	4458	C>T
TaA	4468	C>A
TgA	4468	C>g
tAA	4470	G>T
tAA	4473	A>T
TaA	4483	T>A
TgA	4483	T>g
TaA	4489	C>A
TgA	4489	C>g
tAG	4491	C>T
tAA	4494	A>T
tAA	4503	G>T
TAa	4508	C>A
TAg	4508	C>G
tAG	4518	C>T

tAA	4527	G>T
tAG	4545	A>T
tAG	4551	G>T
tAA	4578	A>T
tAA	4584	A>T
tAA	4587	G>T
tGA	4593	G>T
tAA	4599	G>T
TaA	4606	C>A
TgA	4606	C>g
tAA	4617	A>T
TGa	4622	C>A
TaA	4627	C>A
TgA	4627	C>g
TaA	4630	T>A
TgA	4630	T>g
TaG	4642	G>A
TGa	4643	G>A
TAa	4646	C>A
TAg	4646	C>G
TGa	4658	C>A
tAG	4671	C>T
tGA	4677	A>T
TAa	4685	C>A
TAg	4685	C>G
tAA	4692	C>T
tAG	4695	G>T
tAG	4698	G>T
tAG	4707	A>T
tAG	4722	G>T
tAG	4725	G>T
tAA	4728	C>T
tAG	4731	C>T
tAA	4737	G>T
tAG	4740	G>T
TaG	4759	T>A
tAA	4764	G>T
TAa	4775	C>A
TAg	4775	C>G

TaG	4777	T>A
tAA	4785	C>T
tAG	4794	G>T
tGA	4797	G>T
TAa	4808	C>A
TAg	4808	C>G
tAA	4812	G>T
tGA	4818	G>T
tAA	4845	G>T
tAA	4860	G>T
tGA	4866	A>T
tAG	4875	G>T
TaA	4879	C>A
TgA	4879	C>g
TaA	4906	C>A
TgA	4906	C>g
TaG	4918	T>A
tAA	4920	A>T
tAA	4929	C>T
TaG	4933	T>A
tAA	4935	A>T
tAA	4944	G>T
tAG	4953	C>T
TAa	4994	T>A
TAg	4994	T>G
tAA	5004	G>T
tAA	5007	G>T
tAG	5022	G>T
tAG	5025	A>T
tAA	5031	G>T
TaG	5035	T>A
TaA	5044	C>A
TgA	5044	C>g
tAA	5049	G>T
tAA	5061	A>T
tGA	5064	A>T
tAA	5097	G>T
tAA	5100	G>T
TAa	5117	C>A

TAg	5117	C>G
tAG	5118	A>T
tGA	5127	A>T
tAA	5130	A>T
TaA	5146	T>A
TgA	5146	T>g
tAA	5163	G>T
tAG	5166	G>T
tAA	5187	A>T
tAG	5199	G>T
TGa	5210	T>A
tAA	5211	G>T
tAA	5223	A>T
TAa	5228	T>A
TAg	5228	T>G
tGA	5235	G>T
tGA	5244	G>T
tGA	5247	G>T
tAA	5250	A>T
TaG	5254	G>A
TGa	5255	G>A
TAa	5267	T>A
TAg	5267	T>G
TaG	5272	G>A
TGa	5273	G>A
tAG	5280	C>T
tAA	5289	A>T
tAA	5292	G>T
tGA	5295	A>T
tAA	5298	A>T
tAG	5310	G>T
tAA	5322	G>T
tGA	5328	A>T
tGA	5331	G>T
tGA	5346	G>T
tGA	5349	A>T
tAA	5358	C>T
tAG	5367	A>T
tGA	5370	C>T

tGA	5376	A>T
tAA	5379	G>T
tAG	5385•	C>T
tGA	5391	A>T
tAG	5394	A>T
tAA	5412	G>T
TGa	5420	T>A
TGa	5423	C>A
TAa	5426	T>A
TAg	5426	T>G
tAA	5454	C>T
tAA	5460	G>T
TaG	5464	G>A
TGa	5465	G>A
tAG	5472	C>T
TGa	5480	T>A
tAG	5496	A>T
tAG	5499	G>T
TaA	5506	C>A
TgA	5506	C>g
TaA	5509	C>A
TgA	5509	C>g
tAG	5550	C>T
TaG	5563	G>A
TGa	5564	G>A
tAG	5568	G>T
tAG	5595	C>T
TGa	5603	T>A
tAG	5604	G>T
tGA	5622	C>T
tAG	5625	G>T
TaG	5629	G>A
TGa	5630	G>A
TaG	5635	T>A
TAa	5654	C>A
TAg	5654	C>G
tAG	5655	C>T
TGa	5660	C>A
tAG	5661	C>T

tAG	5664	G>T
TAa	5678	C>A
TAg	5678	C>G
tAG	5688	C>T
TAa	5708	C>A
TAg	5708	C>G
TaA	5710	G>A

The genes of the present invention containing nonsense mutations are also defined as BRCA1 genes having the sequence 5' R1-R2-R3 3'; where R1 is the wild type BRCA1 DNA sequence from codon 1 to X-1; R2 is TAA, TAG or TGA; R3 is the wild type BRCA1 DNA sequence from codon X+1 to 1862; and where X = 2 to 1861.

The genes of the present invention containing nonsense mutations are also defined by being capable of specifically hybridizing to an oligonucleotide probe having at least 12 nucleotides in length and having the sequence 5' R1-R2-R3 3'; where R1 contains at its 3' end three nucleotides complementary to codon X-1 of the wild-type BRCA1 gene; R2 = a sequence complementary to TAG, TGA or TAA; R3 contains at its 5' end three nucleotides complementary to codon X+1 of the wild type BRCA1 gene; where X = 2 to 1862 Other oligonucleotide probes complementary to these probes are also acceptable, hybridizing to the antisense strand. The oligonucleotide probe is unable to specifically hybridize to the wild-type BRCA1 gene with the same binding affinity as to the mutant BRCA1 gene.

The present invention also involves frame shift mutations involving insertions or deletions of 1, 2, 4, 5, 7, 8, or any other number which is not 3 or a multiple of 3, nucleotides. Single base deletions of the present invention form one or more stop codons as indicated in the following TABLE 4. The formed in-frame stop codon and the location are provided. Any expressed protein from BRCA1 genes with these types of mutations should be truncated accordingly. It should be recognized that the present invention includes deletions of 3n+1 bases, where n is an integer greater than zero and less than 1862. These larger deletions mutations have stop codons at nucleotide numbers corresponding to the listed stop codon at the nucleotide

number listed. The corresponding nucleotide numbers of the stop codons will be 3n nucleotides smaller than those listed.

TABLE 4
Single Base Deletions

Codon Formed	Nucleotide Number
TAG	183-185
TGA	207-209
TGA	264-266
TGA	249-251
TGA	258-260
TGA	309-311
TAA	321-323
TGA	378-380
TAA	471-473
TAG	420-422
TAA	432-434
TGA	603-605
TAA	507-509
TGA	612-614
TAA	816-818
TAA	654-656
TGA	708-710
TGA	855-857
TGA	1008-1010
TAA	918-920
TAG	1014-1016
TAG	1056-1058
TGA	1032-1034
TAG	1137-1139
TGA	1101-1103
TGA	1143-1145
TAA	1236-1238
TGA	1176-1178
TAG	1200-1202
TGA	1233-1235
TAA	1242-1244

TAG	1296-1298
TAG	1344-1346
TAA	1332-1334
TAA	1365-1367
TAG	1374-1376
TAG	1404-1406
TAG	1395-1397
TAA	1437-1439
TAG	1473-1475
TGA	1443-1445
TAG	1470-1472
TAA	1539-1541
TAA	1548-1550
TAA	1560-1562
TAG	1566-1568
TAA	1593-1595
TAA	1623-1625
TGA	1710-1712
TAG	1647-1649
TAA	1713-1715
TGA	1752-1754
TGA	1755-1757
TAG	1830-1832
TAA	1878-1880
TAA	1890-1892
TAA	1911-1913
TGA	1950-1952
TAA	1926-1928
TAG	1992-1994
TAG	1995-1997
TAA	2010-2012
TAA	2067-2069
TGA	2025-2027
TAA	2067-2069
TGA	2217-2219
TAA	2082-2084
TGA	2217-2219
TAA	2223-2225
TAG	2322-2324

TAA	2256-2258
TAG	2322-2324
TAA	2373-2375
TAG	2409-2411
TAG	2490-2492
TAG	2448-2450
TAG	2490-2492
TGA	2523-2525
TAA	2559-2561
TAG	2652-2654
TAA	2793-2795
TAA	2709-2711
TAA	2793-2795
TAA	2736-2738
TAA	2793-2795
TAG	3114-3116
TGA	2949-2951
TAG	3114-3116
TGA	3099-3101
TAG	3114-3116
TGA	3186-3188
TAA	3138-3140
TGA	3186-3188
TAG	3258-3260
TAA	3240-3242
TAG	3258-3260
TAG	3300-3302
TAG	3333-3335
TGA	3357-3359
TAG	3375-3377
TAA	3441-3443
TAA	3399-3401
TAA	3441-3443
TGA	3426-3428
TAA	3441-3443
TAG	3465-3467
TAG	3456-3458
TAG	3465-3467
TGA	3501-3503

TAG	3516-3518
TAG	3507-3509
TAG	3516-3518
TAG	3579-3581
TAA	3594-3596
TAG	3744-3746
TAA	3819-3821
TAG	3753-3755
TAA	3819-3821
TGA	3906-3908
TAA	3918-3920
TAA	3939-3941
TGA	3927-3929
TAA	3939-3941
TGA	4035-4037
TGA	4017-4019
TGA	4035-4037
TGA	4068-4070
TGA	4089-4091
TGA	4122-4124
TAG	4212-4214
TAG	4143-4145
TAG	4212-4214
TAA	4206-4208
TAG	4212-4214
TAA	4293-4295
TAG	4266-4268
TAA	4293-4295
TGA	4329-4331
TAA	4332-4334
TAG	4359-4361
TAG	4371-4373
TAA	4416-4418
TAA	4482-4484
TAG	4467-4469
TAA	4482-4484
TAA	4512-4514
TAG	4629-4631
TGA	4758-4760

TAA	4644-4646
TGA	4758-4760
TAG	4791-4793
TGA	4917-4919
TAG	4878-4880
TGA	4917-4919
TAA	4905-4907
TGA	4917-4919
TGA	4932-4934
TGA	5013-5015
TAA	4992-4994
TGA	5013-5015
TGA	5034-5036
TGA	5088-5090
TAA	5043-5045
TGA	5088-5090
TAA	5145-5147
TAA	5115-5117
TAA	5145-5147
TAA	5154-5156
TGA	5184-5186
TGA	5220-5222
TAG	5232-5234
TAG	5256-5258
TGA	5274-5276
TGA	5304-5306
TAG	5409-5411
TGA	5493-5495
TAG	5424-5426
TGA	5463-5465
TGA	5616-5618
TGA	5562-5564
TGA	5616-5618
TAG	5643-5645
TGA	5679-5681

Two base deletions of the present invention form stop codons as indicated in the following TABLE 5. The formed in-frame stop codon and its location are provided. Any

expressed protein from BRCA1 genes with these types of mutations should be truncated accordingly. It should be recognized that the present invention includes deletions of 3n+2 bases, where n is an integer greater than zero and less than 1862. These larger deletions mutations have stop codons at nucleotide numbers corresponding to the listed stop codon at the nucleotide number listed. The corresponding nucleotide numbers of the stop codons will be 3n nucleotides smaller than those listed.

TABLE 5
Two Base Deletions

Codon Formed	Nucleotide Number
TGA	141-143
TAA	162-164
TGA	234-236
TGA	183-185
TGA	234-236
TAA	309-311
TGA	249-251
TGA	309-311
TGA	315-317
TAG	354-356
TGA	366-368
TGA	402-404
TAA	378-380
TGA	402-404
TAA	432-434
TGA	414-416
TAA	432-434
TAA	453-455
TGA	462-464
TGA	477-479
TGA	537-539
TAG	507-509
TGA	537-539
TAA	588-590
TGA	657-659

	TGA	669-671
	TGA	678-680
	TAA	690-692
	TAA	693-695
	TGA	759-761
	TAG	705-707
	TGA	759-761
	TAG	708-710
	TGA	759-761
	TGA	795-797
	TGA	771-773
	TGA	795-797
	TGA	804-806
	TGA	825-827
	TAA	843-845
	TAA	846-848
	TGA	849-851
	TGA	864-866
	TAA	855-857
	TGA	864-866
	TGA	879-881
	TAG	906-908
	TAA	900-902
	TAG	906-908
	TGA	972-974
	TAA	918-920
	TGA	972-974
	TAA	996-998
	TGA	1023-1025
	TGA	1032-1034
	TAA	1035-1037
	TAA	1071-1073
	TAA	1089-1091
	TGA	1101-1103
	TGA	1104-1106
	TAG	1107-1109
	TGA	1149-1151
	TGA	1161-1163
	TAA	1179-1181

TGA	1176-1178
TAA	1179-1181
TAG	1209-1211
TGA	1200-1202
TAG	1209-1211
TGA	1218-1220
TAG	1245-1247
TAA	1263-1265
TGA	1266-1268
TGA	1284-1286
TAG	1278-1280
TGA	1284-1286
TGA	1287-1289
TGA	1302-1304
TGA	1305-1307
TGA	1314-1316
TGA	1326-1328
TGA	1347-1349
TAA	1332-1334
TGA	1347-1349
TGA	1368-1370
TGA	1356-1358
TGA	1368-1370
TGA	1377-1379
TGA	1419-1421
TGA	1395-1397
TGA	1419-1421
TGA	1428-1430
TGA	1443-1445
TGA	1449-1451
TAA	1479-1481
TAA	1464-1466
TAA	1479-1481
TGA	1485-1487
TGA	1551-1553
TAG	1512-1514
TGA	1551-1553
TAG	1539-1541
TGA	1551-1553

TGA	1581-1583
TAA	1617-1619
TAA	1629-1631
TAA	1623-1625
TAA	1629-1631
TGA	1659-1661
TGA	1704-1706
TAA	1725-1727
TAA	1764-1766
TAG	1767-1769
TGA	1776-1778
TAA	1782-1784
TGA	1794-1796
TGA	1809-1811
TAA	1821-1823
TGA	1869-1871
TAA	1857-1859
TGA	1869-1871
TAA	1935-1937
TAA	1911-1913
TAA	1935-1937
TAA	1926-1928
TAA	1935-1937
TAG	1944-1946
TGA	1986-1988
TAG	2001-2003
TAA	2019-2021
TGA	2028-2030
TGA	2040-2042
TAG	2043-2045
TAG	2052-2054
TGA	2058-2060
TAA	2130-2132
TAA	2082-2084
TAA	2130-2132
TAA	2160-2162
TGA	2172-2174
TAA	2184-2186
TGA	2193-2195

TGA	2199-2201
TAA	2247-2249
TGA	2265-2267
TAA	2256-2258
TGA	2265-2267
TAA	2271-2273
TAG	2289-2291
TAA	2331-2333
TAA	2340-2342
TAA	2343-2345
TGA	2349-2351
TGA	2397-2399
TAG	2373-2375
TGA	2397-2399
TAG	2415-2417
TGA	2442-2444
TAG	2481-2483
TAG	2448-2450
TAG	2481-2483
TAA	2514-2516
TGA	2541-2543
TAA	2580-2582
TAA	2574-2576
TAA	2580-2582
TAG	2583-2585
TGA	2589-2591
TAA	2604-2606
TGA	2622-2624
TAA	2628-2630
TGA	2667-2669
TGA	2673-2675
TGA	2820-2822
TAA	2700-2702
TGA	2820-2822
TAA	2709-2711
TGA	2820-2822
TAA	2736-2738
TGA	2820-2822
TAA	2793-2795

TGA	2820-2822
TGA	2826-2828
TGA	2856-2858
TAA	2862-2864
TAA	2886-2888
TAA	2925-2927
TGA	2934-2936
TAA	2937-2939
TAG	2949-2951
TAG	2967-2969
TAA	3024-3026
TAG	2991-2993
TAA	3024-3026
TAA	3087-3089
TAG	3051-3053
TAA	3087-3089
TAA	3093-3095
TGA	3099-3101
TGA	3126-3128
TGA	3147-3149
TGA	3165-3167
TAG	3195-3197
TAA	3201-3203
TAA	3204-3206
TAG	3210-3212
TAA	3225-3227
TAA	3249-3251
TAG	3240-3242
TAA	3249-3251
TGA	3252-3254
TAA	3270-3272
TAG	3264-3266
TAA	3270-3272
TGA	3273-3275
TAA	3291-3293
TAG	3285-3287
TAA	3291-3293
TGA	3294-3296
TGA	3309-3311

TAG	3306-3308
TGA	3309-3311
TGA	3312-3314
TAG	3336-3338
TAG	3369-3371
TAA	3357-3359
TAG	3369-3371
TGA	3390-3392
TAA	3399-3401
TAA	3420-3422
TGA	3426-3428
TGA	3435-3437
TAA	3456-3458
TAA	3477-3479
TAA	3510-3512
TGA	3507-3509
TAA	3510-3512
TAG	3534-3536
TGA	3516-3518
TAG	3534-3536
TGA	3558-3560
TGA	3567-3569
TGA	3570-3572
TGA	3582-3584
TGA	3579-3581
TGA	3582-3584
TGA	3588-3590
TAG	3606-3608
TGA	3615-3617
TGA	3621-3623
TAA	3627-3629
TAG	3648-3650
TAG	3675-3677
TAG	3687-3689
TAG	3768-3770
TAG	3723-3725
TAG	3768-3770
TGA	3744-3746
TAG	3768-3770

TGA	3753-3755
TAG	3768-3770
TGA	3771-3773
TGA	3777-3779
TAA	3813-3815
TAG	3843-3845
TAG	3849-3851
TAA	3876-3878
TAG	3912-3914
TAA	3906-3908
TAG	3912-3914
TGA	3921-3923
TAA	3918-3920
TGA	3921-3923
TAA	3930-3932
TAG	3927-3929
TAA	3930-3932
TAG	3972-3974
TGA	3975-3977
TAG	3996-3998
TGA	4020-4022
TAG	4017-4019
TGA	4020-4022
TGA	4101-4103
TGA	4026-4028
TGA	4101-4103
TAA	4080-4082
TGA	4101-4103
TGA	4125-4127
TGA	4146-4148
TGA	4143-4145
TGA	4146-4148
TGA	4149-4151
TAA	4179-4181
TGA	4170-4172
TAA	4179-4181
TGA	4215-4217
TAA	4206-4208
TGA	4215-4217

TGA	4233-4235
TGA	4239-4241
TGA	4254-4256
TGA	4284-4286
TAA	4323-4325
TGA	4353-4355
TAA	4395-4397
TGA	4371-4373
TAA	4395-4397
TGA	4419-4421
TGA	4434-4436
TAG	4497-4499
TGA	4467-4469
TAG	4497-4499
TGA	4500-4502
TGA	4539-4541
TGA	4548-4550
TAG	4563-4565
TAA	4575-4577
TAA	4581-4583
TAA	4614-4616
TGA	4632-4634
TGA	4629-4631
TGA	4632-4634
TAG	4635-4637
TAG	4674-4676
TGA	4641-4643
TAG	4674-4676
TAA	4704-4706
TGA	4713-4715
TGA	4833-4835
TGA	4836-4838
TGA	4842-4844
TGA	4848-4850
TGA	4857-4859
TGA	4977-4979
TAA	4917-4919
TGA	4977-4979
TAA	4932-4934

TGA	4977-4979
TAA	4992-4994
TAA	5148-5150
TAA	5115-5117
TAA	5148-5150
TGA	5160-5162
TGA	5196-5198
TGA	5208-5210
TAG	5259-5261
TAA	5286-5288
TGA	5307-5309
TGA	5313-5315
TGA	5319-5321
TGA	5601-5603
TAG	5400-5402
TGA	5601-5603
TGA	5421-5423
TGA	5601-5603
TAG	5424-5426
TGA	5601-5603
TGA	5634-5636
TAA	5652-5654
TGA	5658-5660
TAG	5706-5708

Deletion mutations in the BRCA1 gene containing a truncating mutation may also be defined as having the sequence 5' R1-R2 3'; where R1 is the wild type BRCA1 DNA sequence from nucleotide number 120 to X; R2 contains the wild type BRCA1 DNA sequence from nucleotide number X+Y+1 to 5571, where Y = 3n+1 or 3n+2 where n is an integer of zero or greater; and where X = 123 to 5707.

Alternatively, the mutations may be defined as being specifically hybridizable to an oligonucleotide probe being at least 12 nucleotides in length and having the sequence 5' R1-R2 3'; where R1 contains at its 3' end three nucleotides complementary to nucleotide numbers X-2, X-1 and X the wild-type BRCA1 gene; R2 contains at its 5' end three nucleotides complementary

to nucleotide numbers X+Y+1, X+Y+2, and X+Y+3 of the wild type BRCA1 DNA sequence; where Y = 3n+1 or 3n+2 where n is an integer of zero or greater; and where X = 122 to 5706.

Single base insertions of the present invention form stop codons as indicated in the following TABLE 6. The formed in-frame stop codon and the location are provided. Any expressed protein from BRCA1 genes with these types of mutations should be truncated accordingly. It should be recognized that the present invention includes insertions of 3n+1 bases, where n is an integer greater than zero and less than 1861. These larger insertions mutations have stop codons at nucleotide numbers corresponding to the listed stop codon at the nucleotide number listed. The corresponding nucleotide numbers of the stop codons will be 3n nucleotides larger than those listed.

TABLE 6
Single Base Insertions

Codon Formed	Nucleotide Number
TGA	144-146
TGA	123-125
TGA	144-146
TAA	165-167
TGA	144-146
TAA	165-167
TGA	147-149
TAA	165-167
TAA	156-158
TAA	165-167
TGA	237-239
TAA	165-167
TGA	237-239
TAA	177-179
TGA	237-239
TGA	186-188
TGA	237-239
TAG	189-191
TGA	237-239

TGA	189-191
TGA	237-239
TAG	198-200
TGA	237-239
TGA	198-200
TGA	237-239
TGA	204-206
TGA	237-239
TAA	213-215
TGA	237-239
TGA	216-218
TGA	237-239
TAA	231-233
TGA	237-239
TAG	234-236
TGA	237-239
TGA	234-236
TGA	237-239
TAA	312-314
TGA	237-239
TAA	312-314
TAG	249-251
TAA	312-314
TGA	249-251
TAA	312-314
TAA	252-254
TAA	312-314
TAG	258-260
TAA	312-314
TGA	258-260
TAA	312-314
TAA	267-269
TAA	312-314
TAA	276-278
TAA	312-314
TAA	282-284
TAA	312-314
TAA	285-287
TAA	312-314

TAG	300-302
TAA	312-314
TGA	300-302
TAA	312-314
TAG	309-311
TAA	312-314
TGA	309-311
TAA	312-314
TGA	318-320
TAA	312-314
TGA	318-320
TAA	315-317
TGA	318-320
TAG	357-359
TGA	318-320
TAG	357-359
TAA	327-329
TAG	357-359
TAG	330-332
TAG	357-359
TAG	333-335
TAG	357-359
TGA	342-344
TAG	357-359
TAG	345-347
TAG	357-359
TAG	351-353
TAG	357-359
TGA	369-371
TAG	357-359
TGA	369-371
TGA	405-407
TGA	369-371
TGA	405-407
TGA	372-374
TGA	405-407
TAA	381-383
TGA	405-407
TAG	390-392

TGA	405-407
TGA	390-392
TGA	405-407
TAA	435-437
TGA	405-407
TAA	435-437
TGA	417-419
TAA	435-437
TAA	420-422
TAA	435-437
TGA	420-422
TAA	435-437
TAA	420-422
TAA	435-437
TAG	420-422
TAA	435-437
TAA	426-428
TAA	435-437
TAG	429-431
TAA	435-437
TAA	432-434
TAA	435-437
TGA	432-434
TAA	435-437
TAA	432-434
TAA	435-437
TAG	432-434
TAA	435-437
TAA	456-458
TAA	435-437
TAA	456-458
TAA	444-446
TAA	456-458
TAA	447-449
TAA	456-458
TGA	450-452
TAA	456-458
TAA	453-455
TAA	456-458

TGA	465-467
TAA	456-458
TGA	465-467
TGA	480-482
TGA	465-467
TGA	480-482
TAA	474-476
TGA	480-482
TGA	477-479
TGA	480-482
TGA	540-542
TGA	480-482
TGA	540-542
TAG	498-500
TGA	540-542
TAA	507-509
TGA	540-542
TGA	507-509
TGA	540-542
TAA	507-509
TGA	540-542
TAG	507-509
TGA	540-542
TAG	510-512
TGA	540-542
TAA	513-515
TGA	540-542
TAA	522-524
TGA	540-542
TAG	525-527
TGA	540-542
TAG	537-539
TGA	540-542
TAA	591-593
TGA	540-542
TAA	591-593
TGA	546-548
TAA	591-593
TAA	549-551

TAA	591-593
TGA	564-566
TAA	591-593
TAG	570-572
TAA	591-593
TAG	576-578
TAA	591-593
TGA	660-662
TAA	591-593
TGA	660-662
TAG	606-608
TGA	660-662
TAG	615-617
TGA	660-662
TAA	621-623
TGA	660-662
TAA	642-644
TGA	660-662
TAA	654-656
TGA	660-662
TGA	654-656
TGA	660-662
TAA	654-656
TGA	660-662
TAG	654-656
TGA	660-662
TGA	672-674
TGA	660-662
TGA	672-674
TGA	681-683
TGA	672-674
TGA	681-683
TAA	693-695
TGA	681-683
TAA	693-695
TGA	684-686
TAA	693-695
TAA	696-698
TAA	693-695

TAA	696-698
TGA	762-764
TAA	696-698
TGA	762-764
TAA	705-707
TGA	762-764
TGA	705-707
TGA	762-764
TAA	705-707
TGA	762-764
TAG	705-707
TGA	762-764
TAG	708-710
TGA	762-764
TGA	708-710
TGA	762-764
TAG	711-713
TGA	762-764
TGA	720-722
TGA	762-764
TGA	726-728
TGA	762-764
TAG	756-758
TGA	762-764
TGA	759-761
TGA	762-764
TGA	798-800
TGA	762-764
TGA	798-800
TAG	768-770
TGA	798-800
TGA	774-776
TGA	798-800
TAA	783-785
TGA	798-800
TAA	786-788
TGA	798-800
TAG	795-797
TGA	798-800

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TAA	5289-5291
TAA	5265-5267
TAA	5289-5291
TAG	5265-5267
TAA	5289-5291
TAG	5271-5273
TAA	5289-5291
TGA	5271-5273
TAA	5289-5291
TGA	5310-5312
TAA	5289-5291
TGA	5310-5312
TGA	5292-5294
TGA	5310-5312
TAG	5295-5297
TGA	5310-5312
TAA	5298-5300
TGA	5310-5312
TAA	5307-5309
TGA	5310-5312
TGA	5316-5318
TGA	5310-5312
TGA	5316-5318
TGA	5322-5324
TGA	5316-5318

	TGA	5322-5324
	TGA	5604-5606
	TGA	5322-5324
	TGA	5604-5606
	TAG	5328-5330
	TGA	5604-5606
	TGA	5334-5336
	TGA	5604-5606
	TAA	5343-5345
	TGA	5604-5606
	TAG	5349-5351
	TGA	5604-5606
	TAA	5352-5354
	TGA	5604-5606
	TAA	5367-5369
	TGA	5604-5606
	TAG	5376-5378
	TGA	5604-5606
	TGA	5379-5381
	TGA	5604-5606
	TGA	5388-5390
	TGA	5604-5606
	TAG	5391-5393
	TGA	5604-5606
	TAA	5394-5396
	TGA	5604-5606
	TAG	5403-5405
	TGA	5604-5606
	TGA	5412-5414
	TGA	5604-5606
	TAG	5418-5420
	TGA	5604-5606
	TGA	5418-5420
	TGA	5604-5606
	TAG	5421-5423
	TGA	5604-5606
	TGA	5421-5423
	TGA	5604-5606
	TAA	5424-5426

TGA	5604-5606
TGA	5424-5426
TGA	5604-5606
TAA	5424-5426
TGA	5604-5606
TAG	5424-5426
TGA	5604-5606
TAA	5439-5441
TGA	5604-5606
TGA	5451-5453
TGA	5604-5606
TGA	5460-5462
TGA	5604-5606
TAG	5463-5465
TGA	5604-5606
TGA	5463-5465
TGA	5604-5606
TAG	5478-5480
TGA	5604-5606
TGA	5478-5480
TGA	5604-5606
TAA	5496-5498
TGA	5604-5606
TGA	5499-5501
TGA	5604-5606
TGA	5556-5558
TGA	5604-5606
TAG	5562-5564
TGA	5604-5606
TGA	5562-5564
TGA	5604-5606
TGA	5568-5570
TGA	5604-5606
TGA	5571-5573
TGA	5604-5606
TAA	5574-5576
TGA	5604-5606
TAG	5601-5603
TGA	5604-5606

TGA	5601-5603
TGA	5604-5606
TGA	5625-5627
TGA	5628-5630
TGA	5637-5639
TAG	5640-5642
TAG	5652-5654
TGA	5658-5660
TGA	5664-5666
TGA	5670-5672
TAG	5676-5678
TAG	5700-5702
TAG	5706-5708

Two base insertions of the present invention form stop codons as indicated in the following TABLE 7. The formed in-frame stop codon and the location are provided. Any expressed protein from BRCA1 genes with these types of mutations should be truncated accordingly. It should be recognized that the present invention includes insertions of 3n+2 bases, where n is an integer greater than zero and less than 1861. These larger insertions mutations have stop codons at nucleotide numbers corresponding to the listed stop codon at the nucleotide number listed. The corresponding nucleotide numbers of the stop codons will be 3n nucleotides larger than those listed.

TABLE7
Two Base Insertions

Codon Formed	Nucleotide Number
TAG	123-125
TAA	126-128
TAG	126-128
TGA	126-128
TAA	129-131
TAG	129-131
TGA	129-131
TAG	132-134

TAG	141-143
TAG	144-146
TAG	147-149
TAG	150-152
TAA	156-158
TAG	159-161
TAA	162-164
TAA	165-167
TAG	168-170
TAA	171-173
TAA	177-179
TGA	177-179
TAA	180-182
TAA	183-185
TAG	183-185
TGA	183-185
TAG	186-188
TAA	189-191
TAG	189-191
TGA	189-191
TAA	195-197
TAA	198-200
TAG	198-200
TGA	198-200
TAG	204-206
TAA	207-209
TAG	207-209
TGA	207-209
TAA	210-212
TGA	210-212
TAA	213-215
TAG	216-218
TAG	222-224
TAA	225-227
TAG	225-227
TGA	225-227
TAA	228-230
TAA	231-233
TAA	234-236

TAG	234-236
TGA	234-236
TAG	237-239
TAA	243-245
TAA	246-248
TAG	246-248
TGA	246-248
TAA	249-251
TAG	249-251
TGA	249-251
TAA	252-254
TAA	255-257
TAG	255-257
TGA	255-257
TAA	258-260
TAG	258-260
TGA	258-260
TAA	261-263
TAA	267-269
TGA	267-269
TAA	276-278
TAA	282-284
TGA	282-284
TAA	285-287
TGA	285-287
TAG	288-290
TAA	294-296
TAG	294-296
TGA	294-296
TAA	300-302
TAG	300-302
TGA	300-302
TAA	306-308
TAG	306-308
TGA	306-308
TAA	309-311
TAG	309-311
TGA	309-311
TAA	312-314

TAA	315-317
TGA	315-317
TAG	318-320
TAA	321-323
TAA	324-326
TAA	327-329
TAA	330-332
TAA	333-335
TGA	333-335
TAG	342-344
TAA	345-347
TAA	348-350
TAA	351-353
TGA	351-353
TAA	354-356
TAG	354-356
TGA	354-356
TAA	357-359
TAG	366-368
TAG	369-371
TAG	372-374
TAA	378-380
TAG	378-380
TGA	378-380
TAA	381-383
TGA	381-383
TAA	384-386
TAA	387-389
TAA	390-392
TAG	390-392
TGA	390-392
TAG	393-395
TAA	396-398
TAG	396-398
TGA	396-398
TAG	405-407
TAA	408-410
TAG	411-413
TAA	414-416

TAG	414-416
TGA	414-416
TAG	417-419
TAA	420-422
TAG	420-422
TGA	420-422
TAG	423-425
TAA	426-428
TAA	429-431
TAA	432-434
TAG	432-434
TGA	432-434
TAA	435-437
TAA	438-440
TAG	438-440
TGA	438-440
TAG	441-443
TAA	444-446
TAA	447-449
TAG	450-452
TAA	453-455
TAA	456-458
TAA	459-461
TAG	459-461
TGA	459-461
TAG	465-467
TAA	474-476
TAG	477-479
TAG	480-482
TAG	483-485
TAA	486-488
TAG	486-488
TGA	486-488
TAA	489-491
TAA	492-494
TAA	498-500
TAA	501-503
TAG	504-506
TAA	507-509

TAG	507-509
TGA	507-509
TAA	510-512
TAA	513-515
TAG	519-521
TAA	522-524
TAA	525-527
TAA	537-539
TGA	537-539
TAG	540-542
TAG	546-548
TAA	549-551
TAA	555-557
TAG	555-557
TGA	555-557
TAA	558-560
TAG	558-560
TGA	558-560
TAG	564-566
TAA	567-569
TAA	570-572
TAA	576-578
TAG	579-581
TAA	588-590
TAG	588-590
TGA	588-590
TAA	591-593
TAG	597-599
TAA	600-602
TAG	603-605
TAA	606-608
TGA	606-608
TAA	609-611
TAA	615-617
TGA	615-617
TAA	618-620
TGA	618-620
TAA	621-623
TAA	630-632

TGA	630-632
TAA	642-644
TAA	645-647
TGA	645-647
TAA	648-650
TAG	648-650
TGA	648-650
TAG	651-653
TAA	654-656
TAG	654-656
TGA	654-656
TAA	657-659
TAG	660-662
TAA	663-665
TAG	663-665
TGA	663-665
TAG	666-668
TAA	669-671
TAG	669-671
TGA	669-671
TAG	672-674
TAA	675-677
TAG	675-677
TGA	675-677
TAA	678-680
TAG	678-680
TGA	678-680
TAG	681-683
TAG	684-686
TAA	687-689
TAG	690-692
TAA	693-695
TAA	696-698
TAG	699-701
TAA	702-704
TAA	705-707
TAG	705-707
TGA	705-707
TAA	708-710

TAG	708-710
TGA	708-710
TAA	711-713
TAG	714-716
TAG	717-719
TAG	720-722
TAG	726-728
TAA	729-731
TAG	729-731
TGA	729-731
TAA	732-734
TAG	732-734
TGA	732-734
TAA	738-740
TAA	741-743
TAG	750-752
TAA	753-755
TAA	756-758
TAG	759-761
TAG	762-764
TAA	765-767
TAA	768-770
TAA	771-773
TAG	771-773
TGA	771-773
TAG	774-776
TAA	777-779
TAG	777-779
TGA	777-779
TAG	780-782
TAA	783-785
TAA	786-788
TAG	789-791
TAG	792-794
TAA	795-797
TAG	795-797
TGA	795-797
TAG	798-800
TAA	801-803

TAG	801-803
TGA	801-803
TAA	804-806
TAG	804-806
TGA	804-806
TAG	807-809
TAA	810-812
TGA	810-812
TAG	813-815
TAG	816-818
TAA	819-821
TAA	822-824
TAA	825-827
TAG	828-830
TAA	843-845
TAA	846-848
TAA	849-851
TAG	852-854
TAA	855-857
TAG	855-857
TGA	855-857
TAA	858-860
TGA	858-860
TAA	861-863
TAA	864-866
TAG	867-869
TAA	870-872
TGA	870-872
TAG	876-878
TAG	879-881
TAG	882-884
TAA	885-887
TGA	885-887
TAG	894-896
TAA	897-899
TAA	900-902
TAG	900-902
TGA	900-902
TAG	906-908

TAA	909-911
TAA	912-914
TAG	912-914
TGA	912-914
TAG	915-917
TAA	918-920
TAG	918-920
TGA	918-920
TAA	921-923
TAA	924-926
TAG	924-926
TGA	924-926
TAG	930-932
TAG	933-935
TAA	939-941
TAG	939-941
TGA	939-941
TAG	942-944
TAA	945-947
TAA	948-950
TAA	951-953
TAG	957-959
TAA	960-962
TAA	963-965
TAG	963-965
TGA	963-965
TAA	966-968
TAG	966-968
TGA	966-968
TAG	975-977
TAA	978-980
TGA	978-980
TAA	981-983
TAA	984-986
TAA	987-989
TAG	987-989
TGA	987-989
TAA	990-992
TAG	990-992

TGA	990-992
TAA	996-998
TAA	999-1001
TAG	1002-1004
TAA	1005-1007
TAA	1008-1010
TAA	1011-1013
TGA	1011-1013
TAG	1014-1016
TAG	1017-1019
TAA	1020-1022
TAG	1023-1025
TAG	1026-1028
TAA	1029-1031
TAG	1029-1031
TGA	1029-1031
TAA	1032-1034
TAG	1032-1034
TGA	1032-1034
TAA	1035-1037
TAA	1038-1040
TAA	1041-1043
TAA	1044-1046
TAG	1053-1055
TAA	1056-1058
TAG	1056-1058
TGA	1056-1058
TAG	1059-1061
TAA	1062-1064
TAA	1065-1067
TGA	1065-1067
TAA	1074-1076
TAA	1077-1079
TAA	1080-1082
TAG	1080-1082
TGA	1080-1082
TAG	1083-1085
TAG	1086-1088
TAA	1089-1091

TAA	1092-1094
TAG	1095-1097
TAA	1098-1100
TAA	1101-1103
TAG	1101-1103
TGA	1101-1103
TAA	1104-1106
TAG	1107-1109
TAA	1110-1112
TAA	1116-1118
TGA	1116-1118
TAA	1122-1124
TAA	1125-1127
TAG	1128-1130
TAA	1131-1133
TAA	1134-1136
TAG	1137-1139
TAG	1140-1142
TAA	1146-1148
TGA	1146-1148
TAG	1149-1151
TAG	1152-1154
TAA	1161-1163
TAG	1161-1163
TGA	1161-1163
TAG	1164-1166
TAA	1167-1169
TGA	1167-1169
TAA	1170-1172
TAG	1173-1175
TAA	1176-1178
TAG	1176-1178
TGA	1176-1178
TAA	1179-1181
TGA	1179-1181
TAA	1182-1184
TAA	1188-1190
TGA	1188-1190
TAA	1197-1199

TAG	1197-1199
TGA	1197-1199
TAA	1200-1202
TAG	1200-1202
TGA	1200-1202
TAG	1203-1205
TAA	1206-1208
TGA	1206-1208
TAA	1212-1214
TAG	1215-1217
TAA	1218-1220
TAG	1221-1223
TAG	1224-1226
TAG	1227-1229
TAA	1233-1235
TAG	1233-1235
TGA	1233-1235
TAA	1236-1238
TGA	1236-1238
TAA	1239-1241
TAA	1245-1247
TAA	1248-1250
TAA	1251-1253
TAA	1254-1256
TAA	1260-1262
TGA	1260-1262
TAG	1263-1265
TAA	1266-1268
TAG	1269-1271
TAA	1272-1274
TAG	1272-1274
TGA	1272-1274
TAA	1275-1277
TAG	1275-1277
TGA	1275-1277
TAA	1278-1280
TAG	1278-1280
TGA	1278-1280
TAA	1281-1283

TAA	1284-1286
TAG	1287-1289
TAG	1290-1292
TAA	1296-1298
TAG	1296-1298
TGA	1296-1298
TAG	1299-1301
TAA	1302-1304
TAG	1302-1304
TGA	1302-1304
TAG	1305-1307
TAG	1308-1310
TAA	1311-1313
TAG	1311-1313
TGA	1311-1313
TAG	1317-1319
TAG	1320-1322
TAG	1323-1325
TAA	1326-1328
TAG	1326-1328
TGA	1326-1328
TAG	1329-1331
TAA	1332-1334
TAG	1332-1334
TGA	1332-1334
TAA	1335-1337
TAG	1338-1340
TAA	1341-1343
TAG	1344-1346
TAG	1347-1349
TAG	1350-1352
TAG	1353-1355
TAA	1356-1358
TAG	1356-1358
TGA	1356-1358
TAG	1359-1361
TAG	1362-1364
TAA	1368-1370
TAG	1371-1373

TAG	1374-1376
TAG	1377-1379
TAG	1380-1382
TAA	1383-1385
TAG	1383-1385
TGA	1383-1385
TAA	1386-1388
TAG	1386-1388
TGA	1386-1388
TAG	1389-1391
TAA	1392-1394
TAG	1392-1394
TGA	1392-1394
TAA	1395-1397
TAG	1395-1397
TGA	1395-1397
TAG	1398-1400
TAA	1401-1403
TGA	1401-1403
TAA	1404-1406
TAG	1407-1409
TAA	1410-1412
TAG	1410-1412
TGA	1410-1412
TAG	1416-1418
TAA	1419-1421
TAG	1422-1424
TAG	1431-1433
TAG	1434-1436
TAA	1437-1439
TAG	1437-1439
TGA	1437-1439
TAA	1440-1442
TAA	1443-1445
TAG	1443-1445
TGA	1443-1445
TAA	1446-1448
TAA	1449-1451
TAG	1452-1454

TAA	1455-1457
TAG	1458-1460
TAA	1464-1466
TAG	1464-1466
TGA	1464-1466
TAA	1467-1469
TAA	1470-1472
TAG	1470-1472
TGA	1470-1472
TAG	1473-1475
TAG	1476-1478
TAA	1479-1481
TGA	1479-1481
TAA	1482-1484
TAA	1485-1487
TAG	1488-1490
TAG	1491-1493
TAA	1494-1496
TAA	1497-1499
TAA	1500-1502
TAG	1500-1502
TGA	1500-1502
TAG	1503-1505
TAA	1506-1508
TGA	1506-1508
TAA	1509-1511
TAA	1512-1514
TAG	1512-1514
TGA	1512-1514
TAA	1518-1520
TGA	1518-1520
TAA	1521-1523
TGA	1521-1523
TAG	1524-1526
TAA	1527-1529
TAA	1536-1538
TAA	1539-1541
TAG	1539-1541
TGA	1539-1541

TAA	1542-1544
TAG	1548-1550
TAA	1551-1553
TAG	1554-1556
TAA	1557-1559
TAA	1563-1565
TAA	1566-1568
TAG	1569-1571
TAG	1572-1574
TAA	1575-1577
TAG	1575-1577
TGA	1575-1577
TAG	1578-1580
TAA	1581-1583
TAG	1584-1586
TAA	1593-1595
TGA	1593-1595
TAA	1596-1598
TAG	1602-1604
TAA	1614-1616
TAA	1617-1619
TAA	1620-1622
TAA	1623-1625
TAG	1623-1625
TGA	1623-1625
TAA	1626-1628
TAA	1632-1634
TAA	1635-1637
TAA	1638-1640
TGA	1638-1640
TAA	1644-1646
TAA	1647-1649
TAG	1647-1649
TGA	1647-1649
TAG	1650-1652
TAG	1662-1664
TAG	1665-1667
TAA	1668-1670
TAG	1668-1670

TGA	1668-1670
TAA	1671-1673
TAA	1674-1676
TAA	1677-1679
TGA	1677-1679
TAG	1680-1682
TAG	1683-1685
TAA	1686-1688
TAG	1686-1688
TGA	1686-1688
TAG	1689-1691
TAG	1692-1694
TAA	1698-1700
TAA	1701-1703
TGA	1701-1703
TAG	1707-1709
TAA	1710-1712
TAA	1713-1715
TGA	1713-1715
TAA	1716-1718
TAG	1722-1724
TAA	1725-1727
TAA	1728-1730
TAA	1734-1736
TAG	1737-1739
TAA	1743-1745
TGA	1743-1745
TAG	1746-1748
TAG	1752-1754
TAA	1755-1757
TGA	1755-1757
TAA	1758-1760
TGA	1758-1760
TAA	1761-1763
TAA	1764-1766
TAA	1767-1769
TAA	1770-1772
TAG	1773-1775
TAG	1779-1781

TAA	1782-1784
TGA	1782-1784
TAA	1785-1787
TAA	1788-1790
TAA	1791-1793
TAG	1794-1796
TAG	1797-1799
TAA	1800-1802
TAG	1800-1802
TGA	1800-1802
TAA	1803-1805
TAA	1809-1811
TGA	1809-1811
TAG	1812-1814
TAA	1815-1817
TGA	1815-1817
TAA	1818-1820
TAA	1824-1826
TAA	1830-1832
TAG	1833-1835
TAA	1836-1838
TAG	1836-1838
TGA	1836-1838
TAG	1842-1844
TAA	1845-1847
TAG	1848-1850
TAA	1851-1853
TAG	1851-1853
TGA	1851-1853
TAG	1854-1856
TAA	1857-1859
TAG	1857-1859
TGA	1857-1859
TAA	1860-1862
TAA	1863-1865
TAA	1866-1868
TGA	1866-1868
TAG	1869-1871
TAG	1872-1874

TAA	1878-1880
TAA	1881-1883
TAA	1884-1886
TAA	1887-1889
TAA	1890-1892
TAA	1893-1895
TAA	1896-1898
TAA	1899-1901
TAG	1902-1904
TAG	1908-1910
TAA	1911-1913
TAG	1911-1913
TGA	1911-1913
TAA	1914-1916
TAA	1917-1919
TAA	1923-1925
TAA	1926-1928
TAG	1926-1928
TGA	1926-1928
TAA	1929-1931
TAG	1932-1934
TAA	1938-1940
TAA	1941-1943
TAA	1944-1946
TGA	1944-1946
TAA	1947-1949
TGA	1953-1955
TAA	1953-1955
TGA	1953-1955
TAA	1956-1958
TGA	1956-1958
TAA	1959-1961
TGA	1959-1961
TAA	1962-1964
TAG	1962-1964
TGA	1962-1964
TAA	1965-1967
TAG	1965-1967
TGA	1965-1967

TAA	1968-1970
TAA	1971-1973
TAA	1977-1979
TAG	1983-1985
TAG	1989-1991
TAG	1995-1997
TAG	1998-2000
TAA	2001-2003
TAA	2004-2006
TAA	2007-2009
TAA	2013-2015
TAA	2022-2024
TAA	2025-2027
TAG	2025-2027
TGA	2025-2027
TAA	2028-2030
TAG	2031-2033
TAA	2034-2036
TAG	2034-2036
TGA	2034-2036
TAA	2040-2042
TAG	2043-2045
TAA	2046-2048
TAA	2049-2051
TAG	2049-2051
TGA	2049-2051
TAA	2052-2054
TAG	2052-2054
TGA	2052-2054
TAA	2055-2057
TAA	2058-2060
TAG	2061-2063
TAG	2064-2066
TAA	2067-2069
TGA	2067-2069
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TAA	4158-4160
TAG	4161-4163
TAA	4164-4166
TAG	4167-4169
TAA	4170-4172
TAG	4170-4172
TGA	4170-4172
TAG	4173-4175
TAG	4176-4178
TAA	4179-4181

TAA	4182-4184
TAG	4188-4190
TAG	4191-4193
TAA	4197-4199
TAA	4200-4202
TAG	4203-4205
TAA	4206-4208
TAG	4206-4208
TGA	4206-4208
TAA	4209-4211
TAA	4212-4214
TAG	4212-4214
TGA	4212-4214
TAG	4215-4217
TAG	4218-4220
TAG	4221-4223
TAG	4224-4226
TAA	4227-4229
TAG	4227-4229
TGA	4227-4229
TAG	4230-4232
TAA	4233-4235
TAG	4233-4235
TGA	4233-4235
TAG	4236-4238
TAA	4239-4241
TGA	4239-4241
TAG	4242-4244
TAA	4245-4247
TAA	4248-4250
TAG	4251-4253
TAA	4254-4256
TAG	4254-4256
TGA	4254-4256
TAG	4257-4259
TAG	4260-4262
TAA	4263-4265
TAG	4263-4265
TGA	4263-4265

TAA	4266-4268
TAG	4266-4268
TGA	4266-4268
TAG	4269-4271
TAA	4275-4277
TAG	4275-4277
TGA	4275-4277
TAA	4278-4280
TAG	4278-4280
TGA	4278-4280
TAA	4284-4286
TGA	4284-4286
TAG	4287-4289
TAA	4290-4292
TAA	4293-4295
TAG	4293-4295
TGA	4293-4295
TAA	4296-4298
TAA	4299-4301
TAA	4308-4310
TGA	4308-4310
TAG	4311-4313
TAA	4314-4316
TAA	4317-4319
TAA	4326-4328
TAA	4332-4334
TGA	4332-4334
TAA	4335-4337
TAG	4347-4349
TAA	4350-4352
TAG	4353-4355
TAG	4356-4358
TAG	4362-4364
TAG	4365-4367
TAG	4368-4370
TAA	4371-4373
TAG	4371-4373
TGA	4371-4373
TAG	4374-4376

TAG	4383-4385
TAA	4386-4388
TGA	4386-4388
TAA	4395-4397
TAG	4395-4397
TGA	4395-4397
TAA	4398-4400
TAA	4401-4403
TAA	4404-4406
TAG	4404-4406
TGA	4404-4406
TAA	4410-4412
TAG	4410-4412
TGA	4410-4412
TAA	4413-4415
TAA	4416-4418
TAA	4419-4421
TAG	4422-4424
TAA	4425-4427
TAG	4425-4427
TGA	4425-4427
TAA	4428-4430
TAG	4428-4430
TGA	4428-4430
TAG	4431-4433
TAG	4437-4439
TAG	4440-4442
TAA	4449-4451
TAG	4455-4457
TAA	4461-4463
TAA	4464-4466
TAA	4467-4469
TAG	4467-4469
TGA	4467-4469
TAG	4470-4472
TAA	4473-4475
TAG	4476-4478
TAG	4479-4481
TAA	4482-4484

TAG	4482-4484
TGA	4482-4484
TAA	4485-4487
TAA	4488-4490
TAG	4488-4490
TGA	4488-4490
TAA	4494-4496
TGA	4494-4496
TAA	4497-4499
TAA	4500-4502
TAG	4503-4505
TAA	4506-4508
TAG	4506-4508
TGA	4506-4508
TAA	4512-4514
TAA	4515-4517
TAA	4521-4523
TGA	4521-4523
TAG	4527-4529
TAG	4530-4532
TAA	4536-4538
TAG	4536-4538
TGA	4536-4538
TAG	4539-4541
TAG	4542-4544
TAA	4545-4547
TAA	4548-4550
TAG	4548-4550
TGA	4548-4550
TAG	4551-4553
TAG	4554-4556
TAA	4557-4559
TAG	4557-4559
TGA	4557-4559
TAG	4560-4562
TAG	4563-4565
TAA	4566-4568
TAA	4569-4571
TAG	4569-4571

TGA	4569-4571
TAA	4572-4574
TAA	4575-4577
TAA	4578-4580
TAA	4581-4583
TAA	4584-4586
TAG	4587-4589
TAG	4593-4595
TAG	4596-4598
TAG	4599-4601
TAA	4602-4604
TAA	4605-4607
TAG	4605-4607
TGA	4605-4607
TAA	4608-4610
TAG	4608-4610
TGA	4608-4610
TAA	4614-4616
TAG	4614-4616
TGA	4614-4616
TAA	4617-4619
TAA	4620-4622
TAG	4620-4622
TGA	4620-4622
TAA	4626-4628
TAG	4626-4628
TGA	4626-4628
TAA	4629-4631
TAG	4629-4631
TGA	4629-4631
TAG	4632-4634
TAG	4635-4637
TAA	4638-4640
TAA	4641-4643
TAG	4641-4643
TGA	4641-4643
TAA	4644-4646
TAG	4644-4646
TGA	4644-4646

TAA	4647-4649
TAA	4653-4655
TAA	4656-4658
TAG	4656-4658
TGA	4656-4658
TAA	4659-4661
TAG	4659-4661
TGA	4659-4661
TAG	4662-4664
TAA	4665-4667
TGA	4665-4667
TAA	4674-4676
TGA	4674-4676
TAA	4677-4679
TAA	4680-4682
TAA	4683-4685
TAG	4683-4685
TGA	4683-4685
TAA	4689-4691
TAG	4689-4691
TGA	4689-4691
TAG	4695-4697
TAG	4698-4700
TAA	4704-4706
TAA	4707-4709
TAG	4710-4712
TAG	4713-4715
TAG	4716-4718
TAG	4719-4721
TAG	4722-4724
TAG	4725-4727
TAG	4737-4739
TAG	4740-4742
TAA	4743-4745
TAG	4743-4745
TGA	4743-4745
TAG	4746-4748
TAG	4755-4757
TAA	4758-4760

TAG	4758-4760
TGA	4758-4760
TAA	4761-4763
TGA	4761-4763
TAG	4764-4766
TAA	4767-4769
TAA	4770-4772
TAG	4770-4772
TGA	4770-4772
TAA	4773-4775
TAG	4773-4775
TGA	4773-4775
TAA	4776-4778
TAG	4776-4778
TGA	4776-4778
TAA	4782-4784
TAG	4788-4790
TAG	4794-4796
TAG	4797-4799
TAA	4800-4802
TAA	4806-4808
TAG	4806-4808
TGA	4806-4808
TAG	4812-4814
TAA	4815-4817
TAG	4815-4817
TGA	4815-4817
TAG	4818-4820
TAA	4821-4823
TAA	4824-4826
TAA	4830-4832
TAG	4830-4832
TGA	4830-4832
TAA	4833-4835
TAG	4833-4835
TGA	4833-4835
TAG	4836-4838
TAG	4839-4841
TAG	4845-4847

TAA	4848-4850
TAG	4848-4850
TGA	4848-4850
TAG	4851-4853
TAA	4857-4859
TAG	4857-4859
TGA	4857-4859
TAG	4860-4862
TAG	4863-4865
TAA	4866-4868
TAG	4869-4871
TAG	4875-4877
TAA	4878-4880
TAG	4878-4880
TGA	4878-4880
TAG	4881-4883
TAG	4887-4889
TAG	4890-4892
TAA	4893-4895
TAA	4896-4898
TAA	4902-4904
TAG	4902-4904
TGA	4902-4904
TAA	4905-4907
TAG	4905-4907
TGA	4905-4907
TAA	4908-4910
TAA	4911-4913
TAG	4911-4913
TGA	4911-4913
TAG	4914-4916
TAA	4917-4919
TAG	4917-4919
TGA	4917-4919
TAA	4920-4922
TGA	4920-4922
TAG	4923-4925
TAA	4932-4934
TAG	4932-4934

TGA	4932-4934
TAA	4935-4937
TGA	4935-4937
TAG	4938-4940
TAG	4941-4943
TAG	4944-4946
TAA	4947-4949
TAG	4947-4949
TGA	4947-4949
TAG	4950-4952
TAG	4956-4958
TAG	4962-4964
TAG	4965-4967
TAG	4968-4970
TAA	4974-4976
TAA	4977-4979
TAG	4980-4982
TAA	4983-4985
TAG	4986-4988
TAG	4989-4991
TAA	4992-4994
TAG	4992-4994
TGA	4992-4994
TAA	4995-4997
TAG	4998-5000
TAA	5001-5003
TAG	5004-5006
TAG	5007-5009
TAA	5010-5012
TAG	5013-5015
TAA	5016-5018
TGA	5016-5018
TAA	5019-5021
TAG	5022-5024
TAA	5025-5027
TGA	5025-5027
TAG	5031-5033
TAA	5034-5036
TAG	5034-5036

TGA	5034-5036
TAA	5037-5039
TGA	5037-5039
TAG	5040-5042
TAA	5043-5045
TAG	5043-5045
TGA	5043-5045
TAA	5046-5048
TAG	5049-5051
TAA	5052-5054
TAG	5055-5057
TAA	5058-5060
TAA	5061-5063
TAA	5064-5066
TAA	5067-5069
TAA	5070-5072
TAG	5070-5072
TGA	5070-5072
TAA	5073-5075
TAG	5076-5078
TGA	5091-5093

Insertion mutant BRCA1 genes containing a truncating mutation may also be defined as having the sequence 5' R1-R2-R3 3'; where R1 is the wild type BRCA1 DNA sequence from nucleotide number 120 to X; R2 is 3n+1 or 3n+2 nucleotides of any sequence where n is an integer of zero or greater; R3 contains the wild type BRCA1 DNA sequence of nucleotide number X+1 to 5711, and where X = 123 to 5707.

Alternatively, an insertion mutant BRCA1 gene or fragment thereof containing a truncating mutation is capable of specifically hybridizing to an oligonucleotide probe being at least 9 nucleotides in length and having the sequence 5' R1-R2-R3 3'; where R1 contains at its 3' end three nucleotides complementary to nucleotide numbers X-2, X-1 and X of the wild-type BRCA1 gene; R2 = an oligonucleotide having Y nucleotides of any sequence; R3 contains at its 5' end three nucleotides complementary to nucleotide numbers X+1, X+2 and X+3 of the wild

type BRCA1 gene; where Y is 3n+1 or 3n+2 where n is an integer of zero to 1861, and where X = 122 to 5707.

It should be recognized that for TABLES 4-7, each line indicating a stop codon represents several different mutations, each one of which creates the same truncating stop codon. For example, in TABLE 4, the line indicated by TGA stop codon at 207-209 represents twenty-four different mutations, 185delA, 186delG, 187delA, 188delG, 189delT, 190delG, 191delT, 192delC, 193delC, 194delC, 195delA, 196delT, 197delC, 198delT, 199delG, 200delT, 201delC, 202delT, 203delG, 204delG, 205delA, 206delG, 207delT, 208delT. The corresponding mutations for each line in each table are easily determined by anyone of very modest skill in the art knowing only the BRCA1 sequence such as given SEQ ID NO:1 and TABLES 4-7.

A substantially complete listing of all of the mutations, their sites etc. of the present invention is described in Appendix A, B, C, D and E. Likewise, the choice of mutations and corresponding oligonucleotides may be chosen from and determined by the list in Appendix A-E.

An alternative method for defining the mutations of the present invention is by their nucleotide numbers. Mutant BRCA1 genes having nonsense mutations may be described as having the nucleotide sequence R4-R5-R6, where R4 is nucleotide numbers 120 to 3X of the BRCA1 gene; R5 is TAG, TAA or TGA; and R6 is nucleotide numbers 3X+4 to 5711 of the BRCA1 gene; where X is 41 to 1903.

Mutant BRCA1 genes having deletion mutations may be described as having the nucleotide sequence R4-R5, where R4 is nucleotide numbers 120 to Y of the BRCA1 gene; and R5 is nucleotide numbers Y+Z+1 to 5711 of the BRCA1 gene; where Y is 124 to 5707, and Z is 3n+1 or 3n+2 where N is an integer of zero or greater.

Mutant BRCA1 genes having insertion mutations may be described as having the nucleotide sequence R4-R5-R6, where R4 is nucleotide numbers 120 to Y of the BRCA1 gene; R5 is 3n+1 or 3n+2 nucleotides of any sequence where n is an integer of zero or greater; and R6 is nucleotides Y+1 to 5711; wherein Y is from 122 to 5707.

While the present invention encompasses genes with numerous mutations in the BRCA1 gene, applicant reserves the right to lessen the scope and number of mutations to be included in the present invention.

It should be recognized that mutations causing truncations which form a smaller protein molecule than mutations causing truncations of known mutations associated with cancer are expected to also be associated with cancer. Removing additional amino acids from a non-function protein is also believed to result in a non-functional protein.

Useful oligonucleotides according to the present invention are those which will specifically hybridize to BRCA1 sequences in the region of the mutations. The oligonucleotides of the present invention are preferably "biologically active" with respect to structural attributes, such as the capacity of a nucleic acid to hybridize to another nucleic acid molecule or to be used by a polymerase as a primer. Alternatively, such attributes may be catalytic, and thus involve the capacity of the agent to mediate a chemical reaction or response. Typically these oligonucleotides are about 13 to 27 nucleotides in length (longer for large insertions) and have the nucleotide sequence corresponding to the region of the mutations at their respective nucleotide locations on the BRCA1 sequence. Such molecules can be labeled, according to any technique known in the art, such as with radiolabels, fluorescent labels, enzymatic labels, sequence tags, biotin, other ligands, etc.

According to another aspect of the invention, the oligonucleotides contain one or more of the specific mutations constituting DNA probes. Generally it is preferred for each DNA probe to encompass only one mutation. Such molecules may be labeled and can be used as allele-specific oligonucleotide probes to detect the mutation of interest.

Alternatively, the oligonucleotide may be one primer of a PCR primer pair, which upon annealing, will amplify a product. In the situation wherein the target DNA sample does not contain a sequence complementary to the oligonucleotide, annealing does not occur, and thus amplification of a product does not occur.

Polynucleotide-containing biological samples, such as blood, can be tested to determine whether the BRCA1 gene contains one of the specific mutations listed above. To amplify the BRCA1 gene, one may use PCR using primers which hybridize to the ends of the exons or to the introns flanking the exons. To detect mutations in the introns, primers amplifying the introns, especially the regions adjacent to the exons (particularly the splice site regions), may be used. Examples of suitable primers are given in Friedman *et al.*, Nat. Genetics, 8:399-404 (1994).

Amplification may also be performed by a number of other techniques, such as by cloning the gene or gene fragments, and linking the BRCA1 gene or fragments thereof in the sample to a vector. "Shot gun" cloning is particularly preferred. For the purposes of this application, a vector may be any polynucleotide containing system which induces replication such as a plasmid, cosmid, virus, transposon, or portions thereof.

In one embodiment of the invention, the BRCA1 gene or A DNA fragment complementary to its coding sequence is ligated to a vector which is placed inside a suitable host cell or other system for replicating the vector. After replication, the BRCA1 gene or its fragments are then separated from the vector, e.g. by restriction endonuclease digestion, to amplify the copy number of BRCA1 in a particular preparation.

Probes are synthesized to specifically hybridize to any of the list of mutations in TABLES 3-7. On each side of the mutation, the probe overlaps at least 3 nucleotides so that the probes specifically hybridize to a DNA with the mutation. Likewise for probes specific to the mutation site with a sequence complementary for the wild type DNA sequence. By using either or both (if the sample is heterozygous) of these probes which differentially hybridize to mutant and wild-type BRCA1 sequences, one can determine the presence or absence of a mutant BRCA1 gene. Probes which hybridize to the complementary strand of the target DNA may also be used in the same manner.

A pair of isolated allele specific oligonucleotide probes are provided for the mutation 185delAG.

wild-type 5'-AAT CTT AGA GTG TCC CA-3', SEQ ID NO:3
mutant 5'-ATC TTA GTG TCC CAC CT-3', SEQ ID NO:4

SEQ ID NO:3 preferentially may be hybridized to a target BRCA1 sequence under conditions where this probe anneals with a wild type BRCA1 gene or gene fragments, whereas SEQ ID NO:4 preferentially may be hybridized to a target BRCA1 sequence under conditions where this probe anneals with BRCA1 gene or gene fragments containing the 185delAG mutation.

A pair of isolated allele specific oligonucleotide probes are provided for the mutation 1136insA.

wild-type 5'-CAG AAA AAA AGG TAG AT-3', SEQ ID NO:5
mutant 5'-CAG AAA AAA AAG GTA GA-3', SEQ ID NO:6

SEQ ID NO:5 preferentially may be hybridized to a target BRCA1 sequence under conditions where this probe anneals with a wild type BRCA1 gene or gene fragments, whereas SEQ ID NO:6 preferentially may be hybridized to a target BRCA1 sequence under conditions where this probe anneals with BRCA1 gene or gene fragments containing the 1136insA mutation.

A pair of isolated allele specific oligonucleotide probes are provided for the mutation 5382insC.

wild-type 5'-AGA GAA TCC CAG GAC AG-3', SEQ ID NO:7
mutant 5'-AGA GAA TCC CCA GGA CA-3', SEQ ID NO:8

SEQ ID NO:9 preferentially may be hybridized to a target BRCA1 sequence under conditions where this probe anneals with a wild type BRCA1 gene or gene fragments, whereas SEQ ID NO:10 preferentially may be hybridized to a target BRCA1 sequence under conditions

where this probe anneals with BRCA1 gene or gene fragments containing the 5382insC mutation.

A pair of isolated allele specific oligonucleotide probes are provided for the mutation C4446T.

wild-type	5'-AGG ACC TGC GAA ATC CA-3', SEQ ID NO:9
mutant	5'-AGG ACC TGT GAA ATC CA-3', SEQ ID NO:10

SEQ ID NO:11 preferentially may be hybridized to a target BRCA1 sequence under conditions where this probe anneals with a wild type BRCA1 gene or gene fragments, whereas SEQ ID NO:12 preferentially may be hybridized to a target BRCA1 sequence under conditions where this probe anneals with BRCA1 gene or gene fragments containing the C4446T mutation. Comparable probes can be prepared for each mutation of the present invention.

These allele specific oligonucleotides are useful in diagnosis of a subject at risk of having cancer. The allele specific oligonucleotides hybridize with a target polynucleotide sequence containing the mutations listed in TABLES 3-7. The probes having a sequence to naturally occurring (wild-type) BRCA1 hybridize preferentially to the wild type sequence and are useful, for example, as controls. The probes complementary to the sequences containing the mutations listed in TABLES 3-7 are designed to hybridize preferentially to the sequences carrying the specified mutant sequence.

The primers of the invention embrace oligonucleotides of sufficient length and appropriate sequence so as to provide initiation of polymerization on a significant number of nucleic acids in the mutated locus. Examples of preferred sequences for the primers of the present invention are given in the references cited above.

Environmental conditions conducive to synthesis of extension products include the presence of nucleoside triphosphates, an agent for polymerization, such as DNA polymerase, and suitable conditions such as temperature, ion composition, ionic strength and pH. The primer is

preferably single stranded for maximum efficiency in amplification, but may be double stranded. If double stranded, the primer is preferably first treated to separate its strands before being used to prepare extension products. The primer must be sufficiently long to specifically prime the synthesis of extension products in the presence of the inducing agent for polymerization. The exact length of primer will depend on many factors, including temperature, buffer, and nucleotide composition. The oligonucleotide primer typically contains 13-20 or more nucleotides, although it may contain fewer nucleotides.

Primers of the invention are designed to be "substantially" complementary to each strand of the genomic locus to be amplified. This means that the primers must be sufficiently complementary to hybridize with their respective strands under conditions which allow the agent for polymerization to perform a polymerase-mediated primer extension reaction. In other words, the primers should have sufficient complementarity with the 5' and 3' sequences flanking the mutation to hybridize therewith and permit amplification of the genomic locus. "Substantially" the same as it refers to oligonucleotide sequences which have the functional ability to hybridize or anneal with sufficiently stringent conditions to generate sufficient specificity to distinguish between the presence or absence of the mutation. This is measurable by the temperature of melting being sufficiently different to permit easy identification of whether the oligonucleotide is binding to the normal or mutant BRCA1 gene sequence. Conventional stringency conditions are described, for example, by Sambrook *et al*, Molecular Cloning, a laboratory Manual, 2nd Edition, Cold Spring Harbor Press, Cold Spring Harbor, New York (1989), more recent editions and Haymes, *et al.*, Nucleic Acid Hybridization, A Practical Approach, IRL Press, Washington, DC (1985).

Oligonucleotide primers of the invention are employed in the amplification process, which is an enzymatic chain reaction that preferably produces exponential quantities of mutated locus relative to the number of reaction steps involved. Typically, one primer is complementary to the negative (-) strand of the mutated locus and the other is complementary to the positive (+)

strand. Annealing the primers to denatured nucleic acid is generally followed by extension with an enzyme, such as the large fragment of DNA polymerase I (Klenow) and nucleotides, and results in newly synthesized + and - strands containing the target mutated locus sequence. Because these newly synthesized sequences are also templates, repeated cycles of denaturing, primer annealing, and extension results in exponential production of the region (*i.e.,* the target mutated locus sequence) defined by the primers. The product of the chain reaction is a discreet nucleic acid duplex with termini corresponding to the ends of the specific primers employed.

The oligonucleotide primers of the invention may be prepared using any suitable method, such as conventional phosphotriester and phosphodiester methods or automated embodiments thereof. In one such automated embodiment, diethylphosphoramidites are used as starting materials and may be synthesized as described by Beaucage, *et al.*, Tetrahedron Letters, 22:1859-1862, (1981). One method for synthesizing oligonucleotides on a modified solid support is described in U.S. Patent No. 4,458,066.

Any nucleic acid specimen, in purified or non-purified form, can be utilized as the starting nucleic acid or acids, providing it contains, or is suspected of containing, the specific nucleic acid sequence containing the mutated locus. Thus, the process may amplify, for example, DNA or RNA, including messenger RNA, wherein DNA or RNA may be single stranded or double stranded. In the event that RNA is to be used as a template, enzymes, and/or conditions optimal for reverse transcribing the template to DNA would preferably be utilized. In addition, a DNA-RNA hybrid which contains one strand of each may be utilized. A mixture of nucleic acids may also be employed, or the nucleic acids produced in a previous amplification reaction herein, using the same or different primers may be so utilized. The specific nucleic acid sequence to be amplified, *i.e.,* the mutated locus, may be a fraction of a larger molecule or can be present initially as a discrete molecule, so that the specific sequence constitutes the entire nucleic acid. It is not necessary that the sequence to be amplified be present initially in a pure form; it may be a minor fraction of a complex mixture, such as contained in whole human DNA.

DNA utilized herein may be extracted from a body sample, such as blood, tissue material and the like by a variety of techniques such as that described by Maniatis, *et. al.* in *Molecular Cloning:A Laboratory Manual,* Cold Spring Harbor, NY, p. 280-281, 1982). If the extracted sample is impure, it may be treated before amplification with an amount of a reagent effective to open the cells, or animal cell membranes of the sample, and to expose and/or separate the strand(s) of the nucleic acid(s). This lysing and nucleic acid denaturing step to expose and separate the strands will allow amplification to occur much more readily.

The deoxyribonucleotide triphosphates dATP, dCTP, dGTP, and dTTP are added to the synthesis mixture, either separately or together with the primers, in adequate amounts and the resulting solution is heated to about 90°-100°C from about 1 to 10 minutes, preferably from 1 to 4 minutes. This is sufficient to denature any double strands. After this heating period, the solution is allowed to cool at a rate which is preferable for the primer hybridization. To the cooled mixture is added an appropriate agent for effecting the primer extension reaction (called herein agent for polymerization), and the reaction is allowed to occur under conditions known in the art. The agent for polymerization may also be added together with the other reagents if it is heat stable. This synthesis (or amplification) reaction may occur at room temperature up to a temperature above which the agent for polymerization no longer functions. Thus, for example, if DNA polymerase is used as the agent, the temperature is generally no greater than about 40°C. Thermostable DNA polymerases, such as Taq polymerase, may function at a higher temperature.

The agent for polymerization may be any compound or system which will function to accomplish the synthesis of primer extension products, including enzymes. Suitable enzymes for this purpose include, for example, *E. coli* DNA polymerase I, Klenow fragment of *E. coli* DNA polymerase, polymerase muteins, reverse transcriptase, other enzymes, including heat-stable enzymes (*i.e.,* those enzymes which perform primer extension after being subjected to temperatures sufficiently elevated to cause denaturation), such as *Taq* polymerase. The suitable enzyme will facilitate combination of the nucleotides in the proper manner to form the primer

extension products which are complementary to each nucleic acid strand. Generally, the synthesis will be initiated at the 3' end of each primer and proceed in the 5' direction along the template strand, until synthesis terminates, producing molecules of different lengths.

The newly synthesized strand and its complementary nucleic acid strand will form a double-stranded molecule under hybridizing conditions described above and this hybrid is used in subsequent steps of the process. In the next step, the newly synthesized double-stranded molecule is subjected to denaturing conditions using any of the procedures described above to provide single-stranded molecules.

The steps of denaturing, annealing, and extension product synthesis can be repeated as often as needed to amplify the target nucleic acid sequence to the extent necessary for detection. The amount of the specific nucleic acid sequence produced will accumulate in an exponential fashion (PCR. A Practical Approach, ILR Press, Eds. M. J. McPherson, P. Quirke, and G. R. Taylor, 1992).

The amplification products may be detected by Southern blot analysis using non-isotopic detection methods. In such a process, for example, a small sample of DNA containing a very low level of the nucleic acid sequence of the polymorphic locus is amplified, and analyzed via a Southern blotting technique or similarly, using dot blot analysis. The use of non-radioactive probes or labels is facilitated by the high level of the amplified signal. Alternatively, probes used to detect the amplified products can be directly or indirectly detectably labeled, for example, with a radioisotope, a fluorescent compound, a bioluminescent compound, a chemiluminescent compound, a metal chelator or an enzyme. Those of ordinary skill in the art will know of other suitable labels for binding to the probe, or will be able to ascertain such, using routine experimentation. In the preferred embodiment, the amplification products are determinable by separating the mixture on an agarose gel containing ethidium bromide which causes DNA to be fluorescent.

Sequences amplified by the methods of the invention can be further evaluated, detected, cloned, sequenced, and the like, either in solution or after binding to a solid support, by any method usually applied to the detection of a specific DNA sequence such as PCR, oligomer restriction (Saiki, et.al., Bio/Technology, 3:1008-1012, (1985)), allele-specific oligonucleotide (ASO) probe analysis (Conner, et. al., Proc. Natl. Acad. Sci. U.S.A., 80:278, (1983)), oligonucleotide ligation assays (OLAs) (Landgren, et. al., Science, 241:1007, (1988)), and the like. Molecular techniques for DNA analysis have been reviewed (Landgren, et. al., Science, 242:229-237, (1988)).

Preferably, the method of amplifying is by PCR, as described herein and as is commonly used by those of ordinary skill in the art. Alternative methods of amplification have been described and can also be employed as long as the BRCA1 locus amplified by PCR using primers of the invention is similarly amplified by the alternative means. Such alternative amplification systems include but are not limited to self-sustained sequence replication, which begins with a short sequence of RNA of interest and a T7 promoter. Reverse transcriptase copies the RNA into cDNA and degrades the RNA, followed by reverse transcriptase polymerizing a second strand of DNA. Another nucleic acid amplification technique is nucleic acid sequence-based amplification (NASBA) which uses reverse transcription and T7 RNA polymerase and incorporates two primers to target its cycling scheme. NASBA can begin with either DNA or RNA and finish with either, and amplifies to 10^8 copies within 60 to 90 minutes. Alternatively, nucleic acid can be amplified by ligation activated transcription (LAT). LAT works from a single-stranded template with a single primer that is partially single-stranded and partially double-stranded. Amplification is initiated by ligating a cDNA to the promoter oligonucleotide and within a few hours, amplification is 10^8 to 10^9 fold. The QB replicase system can be utilized by attaching an RNA sequence called MDV-1 to RNA complementary to a DNA sequence of interest. Upon mixing with a sample, the hybrid RNA finds its complement among the specimen's mRNAs and binds, activating the replicase to copy the tag-along sequence of interest.

Another nucleic acid amplification technique, ligase chain reaction (LCR), works by using two differently labeled halves of a sequence of interest which are covalently bonded by ligase in the presence of the contiguous sequence in a sample, forming a new target. The repair chain reaction (RCR) nucleic acid amplification technique uses two complementary and target-specific oligonucleotide probe pairs, thermostable polymerase and ligase, and DNA nucleotides to geometrically amplify targeted sequences. A 2-base gap separates the oligonucleotide probe pairs, and the RCR fills and joins the gap, mimicking normal DNA repair. Nucleic acid amplification by strand displacement activation (SDA) typically utilizes a short primer containing a recognition site for *Hinc II* with short overhang on the 5' end which binds to target DNA. A DNA polymerase fills in the part of the primer opposite the overhang with sulfur-containing adenine analogs. *Hinc II* is added but only cuts the unmodified DNA strand. A DNA polymerase that lacks 5' exonuclease activity enters at the cite of the nick and begins to polymerize, displacing the initial primer strand downstream and building a new one which serves as more primer. SDA produces greater than 10^7-fold amplification in 2 hours at 37°C. Unlike PCR and LCR, SDA does not require instrumented Temperature cycling. Another modification of the PCR is the TAQMAN amplification (PERKIN ELMER) where an oligonucleotide is labeled with a fluorescent and a quencher. This oligonucleotide anneals to the target between the primers so that when one primer is extended, the 5' nuclease activity of Taq cleaves of the fluorescent label which is then qualitatively detected and quantitatively determined to correspond to the copy number of amplification. Although PCR is the preferred method of amplification in the invention, other methods such as the above can also be used to amplify the BRCA1 locus in accordance with the present invention.

To sequence the coding region of the BRCA1 gene, each exon is amplified separately using a pair of PCR primers and the resulting PCR products are sequenced in the forward and reverse directions. Any combination of the primers mentioned above which encompass the entire BRCA1 coding region may be used.

An alternative method for determining whether a truncating mutation is present is the Protein Truncation Assay (PTA). Protein truncation assay enables us to identify three types mutations in a truncated BRCA1 protein: nonsense mutation, frame shift mutation, and splice-site mutations. Nonsense mutations (see TABLE 3) result when a single base change in a codon creates a signal to terminate the production of the protein. These signals or stop codons come in three types: TGA, TAA, TAG. Frame shift mutations (see TABLES 4-7) occur when bases are added or deleted from the normal sequence. Thus, disrupting the reading frame of the protein and causing a stop codon downstream from the alteration. Splice-site mutations occurring at the intron/exon boundaries have the potential of causing the deletion of an entire exon. Examples of protein truncation assays for BRCA1 is mentioned in Furnari *et al*, Proceedings of the National Academy of Sciences, U.S.A., 96: p. 12479-12484 (11-1997) and Tashiro *et al*, Cancer Research, 57: 3935-3940 (1997).

Preferably, the Polymerase Chain Reaction (PCR) is performed to amplify the BRCA1 gene copy number. The amplified BRCA1 gene is transcribed and translated in vitro. Detection of truncated proteins is made possible by the use of polyacrylamide gel electrophoresis. The migration of the mutant band on the gel allows for size targeting of the alteration; thus reducing confirmatory sequencing to a minimum.

In another embodiment of the invention, a method is provided for diagnosing a subject having a predisposition or higher susceptibility to cancer, or other pathology associated with BRCA1 mutations, comprising sequencing a target nucleic acid of a sample from a subject by dideoxy sequencing following amplification of the target nucleic acid. In such an embodiment, one does not even need to use any of the oligonucleotides, either primers or probes as described herein. The BRCA1 gene, or fragments thereof, may be directly cloned and then sequenced (such as by dideoxy methods) to determine the presence of absence of a mutation. In such a situation, one need only compare the sequence obtained to a naturally occurring (wild type) BRCA1 gene, or a portion thereof.

In another embodiment of the invention a method is provided for diagnosing a subject having a predisposition or higher susceptibility to cancer comprising contacting a target nucleic acid of a sample from a subject with a reagent that detects the presence of one of the mutations of the present invention and detecting the mutation.

In yet another embodiment of the invention, a method is provided for determining whether either gene therapy or protein therapy (with normal BRCA1 protein) is appropriate for the prevention or treatment of cancers and other BRCA1 related syndromes. For this method, BRCA1 mutations are assayed for in a biological sample for BRCA1 mutations. When present, the use of gene therapy or protein therapy to prevent cancer in the individual is appropriate. Likewise when BRCA1 mutations are found in tumor cells from a patient, gene therapy or protein therapy is appropriate for that individual.

In another embodiment of the invention, a method and reagents are provided for repairing the gene mutation in at least some cells by applying an oligomer comprising the sequence of the wild-type probes to repair the individual's genome by triple strand hybridization. See U.S. Patents 5,650,316 and 5,624,803 for example. This is a form of gene therapy to correct the defect in either apparently normal tissue or in an active tumor. Gene repair may also be performed on excised tumor cells which may be helpful in determining the preferred therapy to be used, particularly the reagents used for gene therapy. Other forms of gene therapy, such as providing a complete copy of a normal BRCA1 gene may also be used. Some gene therapy techniques specific to BRCA1 are discussed in Furnari *et al*, Proceedings of the National Academy of Sciences, U.S.A., 96: p. 12479-12484 (11-1997).

Since the method of the present invention may be applied to detect a mutant BRCA1 gene in a fetus, therapeutic or preventative measures may be possible. Screening of eggs or sperm from heterozygous individuals may permit one to selectively conceive a zygote without the mutant BRCA1 gene since only one half of the sperm or eggs will contain the mutation.

In another embodiment of the invention a method is provided for characterizing a tumor. Histologic type, morphologic grade, differences between inherited and sporadic cancer appear to be distinguished. One method comprises sequencing the target nucleic acid isolated from the tumor or other biological sample to determine if the mutation is present. Sanger, *et al.*, J. Mol. Biol. 142:1617 (1980).

Characterizing a tumor as having originated from an inherited gene, a known or suspected cause, or a sporadic cancer gene may be clinically significant as the prevalence of bilateral breast cancer is higher in individuals with a known mutation in a tumor suppressor gene than in sporadic cases. Weber, Scientific American, JAN-FEB p. 12-21 (1996). The tumor may be classified based on tissue taken from the tumor itself or from a non-tumor site which contains DNA.

Yet another embodiment of the present invention is an isolated mutant BRCA1 DNA sequence which may be the entire sequence, an intron, an exon thereof or a fragment or combination thereof. The BRCA1 DNA may be hybridized to an oligonucleotide probe, primer or polynucleotide and still be considered "isolated". The DNA sequence must contain at least one mutation from the list provided in TABLES 3-7. Preferably, the isolated DNA sequence contains a sequence complementary to at least one of the oligonucleotides complementary to the mutations listed in TABLES 3-7. However, the DNA sequence may contain the DNA sequence of these oligonucleotides. This sequence alone has usefulness or after cloning and expression to determine suitable treatments to prevent formation of a tumor, prevent transmission of the mutant gene to offspring or to decide other prophylactic, diagnostic and treatment protocols. The isolated DNA sequence may also be used for drug design by protein replacement, protein mimetics, screening known and unknown compounds, anti-idiotype antibodies to the BRCA1 active site, for the preparation of an immunogen or vaccine and determining appropriate gene therapy to counter the pathology associated with the mutant BRCA1 gene. For diagnostic

purposes, knowing the mutant BRCA1 sequence for comparison purposes is the critical step in diagnosis.

Another method comprises contacting a target nucleic acid of a sample from a subject with a reagent that detects the presence of the mutation and detecting the mutation. A number of hybridization methods are well known to those skilled in the art. Many of them are useful in carrying out the invention.

The materials for use in the method of the invention are also ideally suited for the preparation of a diagnostic kit. Such a kit may comprise a carrier means being compartmentalized to receive in close confinement one or more container means such as vials, tubes, and the like, each of the container means comprising one or more of the separate elements to be used in the method. For example, one of the container means may comprise means for amplifying BRCA1 DNA, said means comprising the necessary enzyme(s) and oligonucleotide primers for amplifying said target DNA from the subject. Another container may contain oligonucleotide probes for detecting the presence or absence of a mutation.

The oligonucleotide primers include primers having a sequences referenced above or primer sequences substantially complementary or substantially homologous thereto. Other primers flanking the BRCA1 locus or a region containing one of the mutation sites may be used. The target flanking 5' and 3' polynucleotide sequence include other oligonucleotide primers for amplifying the BRCA1 locus will be known or readily ascertainable to those of skill in the art. See the GENBANK sequences mentioned above where flanking sequences are given.

Oligonucleotide probes including probes having substantially the sequence complementary to the mutations listed in TABLES 3-7 or complementary sequences are useful. Other oligonucleotide probes which hybridize to one or more of the BRCA1 mutation sites and sequences substantially complementary or homologous thereto may be used. Other oligonucleotide probes for detecting the mutations will be known or readily ascertainable to those of skill in the art.

The following definitions are provided for the purpose of understanding this invention.

The term "primer" as used herein refers to a sequence comprising two or more deoxyribonucleotides or ribonucleotides, preferably more than three, and more preferably more than eight and most preferably at least 20 nucleotides of the BRCA1 gene wherein the sequence corresponds to a sequence flanking one of the mutations or wild type sequences of BRCA1 corresponding to the mutation sites. Primers may be used to initiate DNA synthesis via the PCR. Oligonucleotides of the present invention can be used for primer hybridization and others will be known or readily ascertainable to those of skill in the art.

The term "substantially complementary to" or "substantially the sequence" refers to sequences which hybridize to the sequences provided under stringent conditions and/or sequences having sufficient homology with, such that the allele specific oligonucleotides of the invention hybridize to the sequence.

"Isolated" as used herein refers to being substantially free of other proteins, lipids, carbohydrates or other materials with which they may be associated. It also refers to being substantially free of polynucleic acids being covalently bound thereto. A DNA may be hybridized to another DNA and still be considered "isolated", such as being hybridized to a solid phase bound or labeled oligonucleotide probe. Such association is typically either in cellular material or in a synthesis medium.

"Biological sample" refers to a polynucleotide containing sample originally from a biological source. The sample may be from a living, dead, paraffin-embedded tumor specimen or even archeological source from a variety of tissues and cells. Examples include: body fluid [blood (leukocytes), urine (epithelial cells), saliva, cervical and vaginal secretions, milk...] skin, hair roots/follicle, mucus membrane (e.g. buccal or tongue cell scrapings), cervicovaginal cells (from PAP smear, etc.) internal tissue (normal or tumor), chorionic villus tissue, amniotic cells, placental cells, fetal cells, cord blood, sperm or egg.

"Coding sequence" or "DNA coding sequence" refers to those portions of a gene which, taken together, code for a peptide (protein), or for which the nucleic acid itself has function. The DNA coding sequence generally encodes the "complete" protein which is one which has the same biological activity as the naturally occurring BRCA1 protein.

A "target polynucleotide" refers to the nucleotide sequence of interest *e.g.,* the BRCA1 encoding polynucleotide. The nucleotides may be deoxyribonucleotides, ribonucleotides, acyclic derivatives and other functional equivalents such as spacer molecules (inosine, the sugar moiety without a base, etc.) and other molecules which are incorporated by a RNA polymerase, a DNA polymerase or a reverse transcriptase.

"Consensus" means the most commonly occurring in the population.

As used herein, a nucleic acid molecule is the "complement" of another nucleic acid molecule if it exhibits complete complementarity. As used herein, molecules are said to exhibit "complete complementarity" when every nucleotide of one of the molecules is complementary to a nucleotide of the other. Two molecules are said to be "substantially complementary" if they can hybridize to one another with sufficient stability to permit them to remain annealed to one another under at least conventional "high-stringency" conditions. Similarly, the molecules are said to be "partially complementary" if they can hybridize to one another with sufficient stability to permit them to remain annealed to one another under conventional "low-stringency" conditions. Conventional stringency conditions are described, for example, by Sambrook, J., et al., (In: Molecular Cloning, a Laboratory Manual, 2nd Edition, Cold Spring Harbor Press, Cold Spring Harbor, New York (1989)), and by Haymes, B.D., et al. (In: Nucleic Acid Hybridization, A Practical Approach, IRL Press, Washington, DC (1985)), both herein incorporated by reference).

As used herein, an oligonucleotide is said to be capable of "specifically hybridizing" to a complementary target polynucleotide if the two molecules are capable of forming an anti-parallel, double-stranded nucleic acid structure under stringent hybridization conditions, whereas

the oligonucleotide is substantially unable to form such a structure when incubated under the same conditions with a target polynucleotide to which the oligonucleotide is not substantially complementary.

"Sequence variation" as used herein refers to any difference in nucleotide sequence between two different oligonucleotide or polynucleotide sequences.

"Polymorphism" as used herein refers to a sequence variation in a gene which is not necessarily associated with pathology.

"Mutation" as used herein refers to an altered genetic sequence which results in the gene coding for a non-functioning protein or a protein with substantially reduced or altered function. Generally, a deleterious mutation is associated with pathology or the potential for pathology. The mutations in the present invention usually involve non-sense and frame shift mutations which cause a truncated (and presumably non-functional) protein to be formed. These truncations are at the terminus of the protein rather than a deletion of one or more amino acids in an internal, non-terminal region of the BRCA1 protein.

The "mutation site" is the location of the added, deleted or substituted bases in the wild-type or consensus BRCA1 DNA sequence which describes the mutant BRCA1 DNA sequence.

"Predetermined sequence variation" as used herein refers to a nucleotide sequence that is designed to be different than the corresponding sequence in a reference nucleotide sequence. A predetermined sequence variation can be a known mutation in a BRCA1 gene.

"BRCA1 gene" refers the published gene sequences, such as those appearing in the GENBANK database under Accession Number, I59546, 2489823, and Y08757. Other different sequences which include polymorphisms and genetic alterations, particularly those which don't cause an amino acid change or which are naturally occurring (wild types), which are not associated with pathology are also considered the BRCA1 gene. The corresponding nucleotides would then be used even if the nucleotide number differs. Generally, the sense strand is referred to. The BRCA1 gene may be in fragments. "Fragments" are segments of the BRCA1 gene,

generally about 15 or more nucleotides in length, usually a few hundred or more nucleotides in length and potentially containing the particular mutation site of interest. The complementary strand to the sense strand of the BRCA1 gene (the so-called antisense strand) is also considered the "BRCA1 gene". While the BRCA1 gene discussed herein is the human BRCA1 gene, the corresponding assays and reagents for the gene in other animals may also be used. The BRCA1 gene includes the coding sequences, non-coding sequences (e.g. introns) and regulatory regions affecting gene expression.

"Allele specific detection assay" as used herein refers to an assay to detect the presence or absence of a predetermined sequence variation in a test polynucleotide or oligonucleotide by annealing the test polynucleotide or oligonucleotide with a polynucleotide or oligonucleotide of predetermined sequence such that differential DNA sequence based techniques or DNA amplification methods discriminate between normal and mutant. Allele Specific Oligonucleotide hybridization is sometimes referred to ASO or the ASO method.

"Sequence variation locating assay" as used herein refers to an assay that detects a sequence variation in a test polynucleotide or oligonucleotide and localizes the position of the sequence variation to a sub-region of the test polynucleotide, without necessarily determining the precise base change or position of the sequence variation.

"Targeted confirmatory sequencing" as used herein refers to sequencing a polynucleotide in the region wherein a sequence variation has been located by a sequence variation locating assay in order to determine the precise base change and/or position of the sequence variation.

"Probe" includes any oligonucleotide which hybridizes to a BRCA1 or mutant BRCA1 sequence. The probe may be labeled (directly or indirectly) or it may act as a primer such as a PCR primer.

"Cancer", "tumor" and "neoplasm" are used interchangeably to refer to certain abnormal cells. The terms are not meant to denote a stage of malignancy.

The invention in several of its embodiments includes:

DETECTION OF PREDETERMINED SEQUENCE VARIATIONS

Stage I analysis is used to determine the presence or absence of a predetermined nucleotide sequence variation; preferably a known mutation or set of known mutations in the test gene. In accordance with the invention, such predetermined sequence variations are preferably detected by allele specific hybridization, a sequence-dependent-based technique which permits discrimination between normal and mutant alleles. An allele specific assay is dependent on the differential ability of mismatched nucleotide sequences (*e.g.*, normal:mutant) to hybridize with each other, as compared with matching (*e.g.*, normal:normal or mutant:mutant) sequences.

DETECTION OF PREDETERMINED SEQUENCE VARIATIONS USING ALLELE SPECIFIC HYBRIDIZATION

A variety of methods well-known in the art can be used for detection of predetermined sequence variations by allele specific hybridization. Preferably, the test gene is probed with allele specific oligonucleotides (ASOs); and each ASO contains the sequence of a known mutation. ASO analysis detects specific sequence variations in a target polynucleotide fragment by testing the ability of a specific oligonucleotide probe to hybridize to the target polynucleotide fragment. Preferably, the oligonucleotide contains the mutant sequence (or its complement). The presence of a sequence variation in the target sequence is indicated by hybridization between the oligonucleotide probe and the target fragment under conditions in which an oligonucleotide probe containing a normal sequence does not hybridize to the target fragment. A lack of hybridization between the sequence variant (*e.g.*, mutant) oligonucleotide probe and the target polynucleotide fragment indicates the absence of the specific sequence variation (*e.g.*, mutation) in the target fragment. In a preferred embodiment, the test samples are probed in a standard dot blot format. Each region within the test gene that contains the sequence corresponding to the ASO is individually applied to a solid surface, for example, as an individual dot on a membrane. Each individual region can be produced, for example, as a separate PCR amplification product

using methods well-known in the art (see, for example, the experimental embodiment set forth in Mullis, U.S. Patent No. 4,683,202). The use of such a dot blot format is described in detail in the Examples below, detailing the Stage I analysis of the human BRCA1 gene to detect the presence or absence of different known mutations using corresponding ASOs.

Membrane-based formats that can be used as alternatives to the dot blot format for performing ASO analysis include, but are not limited to, reverse dot blot, MAD (multiplex amplification assay), and multiplex allele-specific diagnostic assay (MASDA).

In a reverse dot blot format, oligonucleotide or polynucleotide probes having known sequence are immobilized on the solid surface, and are subsequently hybridized with the labeled test polynucleotide sample. In this situation, the primers may be labeled or the NTPs maybe labeled prior to amplification to prepare a labeled test polynucleotide sample. Alternatively, the test polynucleotide sample may be labeled subsequent to isolation and/or synthesis.

In a multiplex format, individual samples contain multiple target sequences within the test gene, instead of just a single target sequence. For example, multiple PCR products each containing at least one of the ASO target sequences are applied within the same sample dot. Multiple PCR products can be produced simultaneously in a single amplification reaction using the methods of Caskey *et al.*, U.S. Patent No. 5,582,989. The same blot, therefore, can be probed by each ASO whose corresponding sequence is represented in the sample dots.

A MASDA format expands the level of complexity of the multiplex format by using multiple ASOs to probe each blot (containing dots with multiple target sequences). This procedure is described in detail in U.S. Patent No. 5,589,330 by A.P. Shuber, and in Michalowsky *et al.*, American Journal of Human Genetics, 59(4): A272, poster 1573 (October 1996), each of which is incorporated herein by reference in its entirety. First, hybridization between the multiple ASO probe and immobilized sample is detected. This method relies on the prediction that the presence of a mutation among the multiple target sequences in a given dot is sufficiently rare that any positive hybridization signal results from a single ASO within the probe

mixture hybridizing with the corresponding mutant target. The hybridizing ASO is then identified by isolating it from the site of hybridization and determining its nucleotide sequence.

Suitable materials that can be used in the dot blot, reverse dot blot, multiplex, and MASDA formats are well-known in the art and include, but are not limited to nylon and nitrocellulose membranes.

When the target sequences are produced by PCR amplification, the starting material can be chromosomal DNA in which case the DNA is directly amplified. Alternatively, the starting material can be mRNA, in which case the mRNA is preferably first reversed transcribed into cDNA and then amplified according to the well known technique of RT-PCR (see, for example, U.S. Patent No. 5,561,058 by Gelfand *et al.*).

The methods described above are suitable for moderate screening of a limited number of sequence variations. However, with the need in molecular diagnosis for rapid, cost effective large scale screening, technologies have developed that integrate the basic concept of ASO, but far exceed the capacity for mutation detection and sample number. These alternative methods to the ones described above include, but are not limited to, large scale chip array sequence-based techniques. The use of large scale arrays allows for the rapid analysis of many sequence variants. A review of the differences in the application and development of chip arrays is covered by Southern, Trends In Genetics, 12: 110-115 (March 1996) and Cheng *et al.*, Molecular Diagnosis, 1:183-200 (Sept. 1996). Several approaches exist involving the manufacture of chip arrays. Differences include, but not restricted to: type of solid support to attach the immobilized oligonucleotides, labeling techniques for identification of variants and changes in the hybridization of the target polynucleotide to the probe.

A promising methodology for large scale analysis on "DNA chips" is described in detail in Hacia *et al.*, Nature Genetics, 14:441-447 (1996), which is hereby incorporated by reference in its entirety. As described in Hacia *et al.*, high density arrays of over 96,000 oligonucleotides, each 20 nucleotides in length, are immobilized to a single glass or silicon chip using light

directed chemical synthesis. Contingent on the number and design of the oligonucleotide probe, potentially every base in a sequence can be interrogated for alterations. Oligonucleotides applied to the chip, therefore, can contain sequence variations that are not yet known to occur in the population, or they can be limited to mutations that are known to occur in the population.

Prior to hybridization with oligonucleotide probes on the chip, the test sample is preferably isolated, amplified and labeled (e.g. fluorescent markers) by means well known to those skilled in the art. The test polynucleotide sample is then hybridized to the immobilized oligonucleotides. The intensity of hybridization of the target polynucleotide to the immobilized probe is quantitated and compared to a reference sequence. The resulting genetic information can be used in molecular diagnosis.

A common, but not limiting, utility of the "DNA chip" in molecular diagnosis is screening for known mutations. However, this may impose a limitation to the technique by only looking at mutations that have been described in the field. The present invention allows allele specific hybridization analysis be performed with a far greater number of mutations than previously available. In accordance with the present invention, DNA chips may be constructed with any number of ASO's specific for any number of mutations of the present invention. Such DNA chips may include hundreds, thousands, or more different ASO's, optionally enabling the screening for all possible mutations of the present invention in a single DNA chip. Preferably, DNA chips of the present invention contain about 10 to about 1000, about 100 to about 1,000, about 1,000 to about 10,000, about 10,000 to about 10,0000, or even greater than 100,000 allele specific oligonucleotides specific for the mutations of the present invention. Additionally, such a DNA chip may optionally contain ASO's specific for those missense mutations for which a clinical significance has been established and/or ASO's specific for wild-type BRCA1 DNA sequences. Thus, the efficiency and comprehensiveness of large scale ASO analysis will be broadened, reducing the need for cumbersome end-to-end sequence analysis, not only with known mutations but in a comprehensive manner all mutations which might occur as predicted

by the principles accepted, and the cost and time associated with these cumbersome tests will be decreased.

EXAMPLE

Genomic DNA (at least about 100 ng) is isolated from white blood cells of a subject with a family history of various cancer. Genomic DNA (at least about 100 ng) is also isolated from a wide variety of fresh tumor cells from biopsy, frozen tumor tissue previously surgically removed and tumor cell lines. Dideoxy sequence analysis is performed following polymerase chain reaction amplification of the BRCA1 gene. The primers are the same as used in the references above.

Each segment of the BRCA1 gene is subjected to direct dideoxy sequence analysis by asymmetric amplification using the polymerase chain reaction (PCR) to generate a single stranded product amplified from this DNA sample. Shuldiner, *et al.*, Handbook of Techniques in Endocrine Research, p. 457-486, DePablo,F., Scanes, C., eds., Academic Press, Inc., 1993. Fluorescent dye is attached for automated sequencing using the TAQ DYE TERMINATOR KIT (PERKIN-ELMER cat# 401628). DNA sequencing is performed in both forward and reverse directions on an APPLIED BIOSYSTEMS, INC. (ABI) automated sequencer (Model 377). The software used for analysis of the resulting data is ASEQUENCE NAVIGATOR@ purchased through ABI.

The methods of the invention, which can be used to detect sequence variations in any polynucleotide sample, are demonstrated in the Example set forth in this section, for the purpose of illustration, for one gene in particular, namely, the human BRCA1 gene. The BRCA1 coding sequence is approximately 5592 base pairs encoding the 403 amino acids BRCA1 protein.

Designing an Allele Specific Oligonucleotide (ASO) Probe

An allele specific oligonucleotide probe is a short, single stranded polynucleotide that is engineered to hybridize exactly to a target sequence under a given set of conditions. Routinely, ASO probes are designed to contain sequences identical to the normal allele and sequence variation respectively. Hybridization of the probe to the target allows for the discrimination of a variant sample. Under stringent conditions, a probe with a variation as simple as a single-base pair will not hybridize to a normal sequence due to a destabilizing effect of the normal-mutant duplex (Ikuta, S. *et al*, Nucleic Acids Research, 15: 797-811 (1987). For use in this invention, probes are used to discriminate between a wild-type or normal sequence from one that is mutated. Each probe pair contains a polynucleotide sequence that encompassed an area that would identify a selected mutation of the BRCA1 gene.

The design of an ASO hybridization probe must meet two basic requirements. (Current Protocols in Human Genetics, 9.4, (1995)). First, probes that are used together in the same pool should be around the same length. Although the standard length of a probe is optimally about 17 base pairs, the range can be as short as about 13 or as long as 30 or more. If the mutation contains a long insertion, a longer probe may be desirable. Second, the mismatched region should not be placed at the end of the probe, but approximately in the middle of the sequence. In addition, the placement of a mismatch, in the case of a longer probe, should not be at the end, but at a position that allows strong hybridization and stabilization of the polynucleotide strand. In order to minimize the effects of variations in base composition of the probes, tetramethylammonium chloride may be used as in the ASO hybrid's buffer (Shuber, U.S. Patent No. 5,633,134). Conventionally, ASO probes are synthesized on a DNA synthesizer. They can be labeled with isotopic or non-isotopic detection agents using means familiar to those of skill in the art. The process outlined in this application for making and using probes can be applicable for other gene sequences.

Protein Truncation Assay

PCR Amplification

BRCA1 is first amplified by PCR from a patient sample using one or more primer sets. A single set of primers may be used to amplify the entire BRCA1 gene or multiple sets of primers may be used. Preferably one need not use a separate set of primers for each exon because the protein expression products are so small that detecting a truncation will be difficult.

Using the primer sets referenced above in a reaction containing Ex Taq Buffer 10X 5.0 mL, dNTP's 2.5mM 4.0 mL, Forward primer 10mM 1.0 mL, Reverse primer 10mM 1.0 mL, TaKaRa Ex Taq (Oncor RR001B) 2.5 U 1.0 mL, Template DNA 100 ng/mL 1.0 mL and OmniPure dH2O to 50 mL final volume. One DNA control (placental DNA), one positive control reaction and three no template control reactions are included for each sample batch..

PCR for the BRCA1 gene is performed using the following thermocycling conditions (4 linked programs):

Temperature	Time	# of Cycles
94°C	5 min.	1
94°C	30 sec.	\
55°C	1 min.	} 35
72°C	3 min.	/
72°C	5 min.	1
4°C	hold	1

5 m\μL of the PCR product is placed on a 2% agarose gel. On the same gel a DNA 100 BP LADDER (Gibco BRL 15628-019) and a low DNA MASS LADDER (Gibco BRL 10068-013) is placed to verify product size.

The resulting product is analyzed according to the following rules: 1)Each patient sample must show a band of the correct size. If a patient sample demonstrates smearing or multiple bands, the PCR reaction needs to be repeated (no more than three times) until a clean, single band is detected. If no PCR product is visible or if only a weak band is visible, but the placental

DNA sample worked well, the sample is reamplified with twice as much template. The volume of the reaction is adjusted appropriately.

All "No template" (2 - 3) reactions must not show amplification products of any size. If any one shows any contamination (i.e. specific amplification product), all PCR products should be thrown away and the entire PCR set-up should be repeated after appropriate PCR decontamination procedures have been taken.

The intensity of the patient sample PCR product is compared with that of the DNA 100 bp ladder. The optimum amount of PCR product on the gel should be 50 - 100 ng. If less than this is present or if the intensity of the patient sample is less than half the intensity of the placental control sample, repeat the PCR reaction until sufficient quantity is obtained. If no PCR product is visible or if only a weak band is visible, but the placental DNA sample worked well, the patient sample is reamplified with twice as much template DNA.

The PCR product is precipitated by adding the following to each tube: 3M sodium acetate 30 μL, 20 mg/mL glycogen 2 μL, dH20 178 μL, and PCR product 90 μL. The reaction is mixed by inverting the tubes 4 - 6 times. 600 μL of 100% ethanol (200 proof) is added to each tube and the reaction can be placed at -20°C overnight. The following day, samples are allowed to equilibrate to room temperature before proceeding. The tubes are centrifuged at 13,000 rpm for 15 minutes at room temperature in an IEC microcentrifuge. The supernatant is removed leaving a pellet. 1 mL cold 70% ethanol is added to each tube and centrifuged at 13,000 rpm for 5 minutes at room temperature in the IEC microcentrifuge. The supernatant is again removed leaving the pellet. The tubes are dried by vacuum for 10 - 15 minutes until no ethanol remains in the tube. Redissolve the pellet in 10 μL of dH2O.

1 μL of the PCR product is electrophoresed on a 2% TAE agarose gel, in parallel with a DNA 100 bp ladder and a DNA mass ladder to verify product size and amount of product. The mass of the 1 μL purified PCR product is estimated using the DNA mass ladder as a reference. The band equals one-tenth of the total quantity of purified PCR product. The amount required

for the lysate reaction is between 500 - 750 ng. For example, if the band is the intensity of the 100 ng marker, then the amount needed for the lysate reaction is 5.0 - 7.5 μL of purified PCR product. If there is less than 500 ng total, the PCR must be repeated.

In vitro transcription/translation

This procedure is performed using the TnT Coupled Reticulocyte Lysate system from Promega L4610. Each kit contains the reagents necessary for 80 25 μL translation reactions. This kit allows the synthesis of the specified protein from PCR product.

The TnT Rabbit Reticulocyte Lysate is thawed in gloved hands and immediately place on ice. The TnT T7 RNA Polymerase and RNase Inhibitor (Boehringer Mannheim 799025) are placed on ice at all times. Solutions are mixed in the following order in a 0.5 mL microcentrifuge tube. Rabbit Reticulocyte Lysate 12.5 μL, TnT Reaction buffer 1.0 μL, TnT T7 RNA Polymerase 0.5 μL, Amino Acid Mixture minus Met (1 mM)0.5 μL, RNase inhibitor 0.5 μL, per 15.0 ml sample.

The following are added to a clean labeled 0.5 mL microcentrifuge tube for each reaction: master mix 15.0 μL, ^{35}S methionine (Amersham SJ1015) (1 mCi/100 μL) 2.0 μL, DNA template (500-750 ng) per μL, sdH2O to 25 μL final volume. The reactions are placed in the 30°C water bath for 1.5 hours.

Gel Preparation, Run and Handling

Pre-run the gel (15% Tris-glycine Ready gel (15 well) BioRad 161-0938 or (10 well) BioRad 161-0908) in a Mini Protean II Gel apparatus (BioRad 165-2941) for 15 minutes to equilibrate the gel with the SDS from the running buffer (10X Tris/Glycine/SDS, BioRad 161-0732). Load samples and check the buffer volume during the course of the run to assure that nothing has leaked out. Decreased buffer volume in the middle chamber will interfere with the current. Monitor the progress of the gel running to prevent the lower marker (blue band) from running off the gel. The average running time is 3 hours.

The gel is rinsed with dH2O after fixing the gel to remove excess salicylic acid and oriented. A mixture of 5.26 mL of beta-mercaptoethanol (BioRad 161-0710) and 94.74 mL of Laemelli sample buffer (BioRad 161-0737) was prepared. The 35S ladder is added as follows in a labeled, 0.5 mL microcentrifuge tube: 7 kD marker in 5 μL, 11 kD marker in 5 μL, 25 kD marker in 2 μL, 74 kD marker in 2 μL. A second labeled tube completes the 35S ladder with: combined lysate 14 μL, sample buffer 32 μL, 100 μM IAA 12 μL, sdH2O 22 mL with a total of 20 μL per gel.

In a 0.5 μL microcentrifuge tube, the following is added:

2 μL lysate reaction, 8 μL sample buffer, 4 μL 100 mM IAA, 6 μL sdH2O, to a total load volume of 20 μL. The samples are heated at 95°C for 3 minutes and then placed on ice for 2 minutes before loading gel. For every gel run, the following controls are included:

a. 10 μL of Broad Range Prestained SDS-PAGE standard (BioRad 161-0318) heated to 37°C to dissolve any precipitated material.

b. 35S ladder

c. one negative control per sample

d. one positive control specific to the fragment under analysis

During the first 20 minutes, the samples are run at 10 mA (20 mA for two gels). This allows the protein to migrate through the resolving gel at a slower rate. After 20 minutes, the current is increased to 20 mA (40 mA for two gels) for the remainder of the gel run. The buffer volume is checked during the course of the run to assure that nothing has leaked out. Decreased buffer volume in the middle chamber will interfere with the current. The average running time is 3 hours. The lower marker on the polypeptide standard is 7.1 kD (blue band) and should not run off the gel.

After running the gel, the plastic adhesive is removed from the back of the ready gel and gently peel off the top plate. The gel will remain attached to the back plate. The gel is placed in fixative solution of Isopropanol 250 mL, sdH2O 650 mL and acetic acid 100 mL in a volume

sufficient to cover gel for 15 minutes with swirling the gel occasionally by hand or on a rotating plate. The gel is then placed in a fluorogenic agent of salicylic acid 160 g and sdH2O to 1 L final volume, pH adjusted to pH 6.0, in a volume sufficient to cover gel for 15 minutes with swirling the gel occasionally by hand or on a rotating plate. The gel is then rinsed with sdH2O to remove any excess salicylic acid.

The gel is dried by sandwiching it between two sheets of cellophane and drying it in a gel dryer (BioRad 165-1771) for 1.5 - 2.5 hours. The dried gel is removed, placed in a film cassette(Fisher IB1502350) taped to a piece of film (Kodak BioMax MR Sigma 870-1302) over the dried gel(s), oriented with Identi-kit tape (Diversified Biotech ID-100) and exposed for 3 - 5 hours at -80°C. The film may be exposed overnight at -20°C or over the weekend at room temperature if needed.

Patient samples are compared to the normal genomic DNA fragments. Truncated proteins are possible at any point along the sequence. Therefore, a shift in bands located in any patient sample is an indication of a mutation. There are areas in the sequence where a mutation can occur that are difficult to detect because of the small molecular weight protein formed by the truncation or because a stop signal occurs at the end of the sequence. These mutations are scanned for as follows. The base pairs at the 5' end of BRCA1 are sequenced. This assures the ability to detect, by means of sequencing, proteins 10 kD or less and the ability to detect, by means of protein truncation, anything greater than 10 kD. A mutation causing a stop codon to occur at the end of the BRCA1 gene is identified by sequencing the final 257 base pairs.

The truncated BRCA1 protein should be of equal intensity on the gel as the normal BRCA1 protein. When a potential truncated band is identified, the protein must be sized using the 35S radiolabeled marker. For example, if a band appears to be in the area of the 50 kD marker, then the range of inspection for the mutation is between 40-50 kD. The position of the stop signal does not always indicate the position where the mutation occurred. Using the

following conversion factor: 270 bp = 10 kD, the molecular weight can be converted into base pairs.

DETAILED METHOD FOR THE DETECTION OF SEQUENCE VARIATIONS IN POLYNUCLEOTIDES

Isolation of Genomic DNA

White blood cells are collected from the patients and genomic DNA is extracted from the white blood cells according to well-known methods (Sambrook, *et al.*, Molecular Cloning, A Laboratory Manual, 2nd Ed., 1989, Cold Spring Harbor Laboratory Press, at 9.16 - 9.19). Genomic DNA is similarly extracted from a wide variety of fresh tumor cells from biopsy, frozen tumor tissue previously surgically removed and tumor cell lines.

PCR Amplification for Sequencing

The genomic DNA is used as a template to amplify a DNA fragment encompassing the site of the mutation to be tested. The 25 µl PCR reaction contains the following components: 1 µl template (100 ng/ ml) DNA, 2.5 µl 10X PCR Buffer (PERKIN-ELMER), 1.5 µl dNTP (2 mM each dATP, dCTP, dGTP, dTTP), 1.5 µl Forward Primer (10 mM), 1.5 µl Reverse Primer (10 mM), 0.5 µl (2.5 U total) AMPLITAQ GOLD™ TAQ DNA POLYMERASE or AMPLITAQ7 TAQ DNA POLYMERASE (PERKIN-ELMER), 1.0 to 5.0 µl (25 mM) $MgCl_2$ (depending on the primer) and distilled water (dH_2O) up to 25 µl. All reagents for each exon except the genomic DNA can be combined in a master mix and aliquoted into the reaction tubes as a pooled mixture.

For each exon analyzed, the following control PCRs are set up:

(1) "Negative" DNA control (100 ng placental DNA (SIGMA CHEMICAL CO., St. Louis, MO)

(2) Three "no template" controls

PCR for all exons is performed using the following thermocycling conditions:

Temperature	Time	Number of Cycles
95°C	5 min. (AMPLITAQ)	1
or	10 min. (GOLD)	
95°C	30 sec. \	
55°C	30 sec. }	30 cycles
72°C	1 min /	
72°C	5 min.	1
4°C	hold	1

Quality control agarose gel of PCR amplification:

The quality of the PCR products is examined prior to further analysis by electrophoresing an aliquot of each PCR reaction sample on an agarose gel. 5 µl of each PCR reaction is run on an agarose gel along side a DNA 100 BP DNA LADDER (Gibco BRL cat# 15628-019). The electrophoresed PCR products are analyzed according to the following criteria:

Each patient sample must show a single band of the size corresponding the number of base pairs expected from the length of the PCR product from the forward primer to the reverse primer. If a patient sample demonstrates smearing or multiple bands, the PCR reaction must be repeated until a clean, single band is detected. If no PCR product is visible or if only a weak band is visible, but the control reactions with placental DNA template produced a robust band, the patient sample should be re-amplified with 2X as much template DNA.

All three "no template" reactions must show no amplification products. Any PCR product present in these reactions is the result of contamination. If any one of the "no template" reactions shows contamination, all PCR products should be discarded and the entire PCR set of reactions should be repeated after the appropriate PCR decontamination procedures have been taken.

The optimum amount of PCR product on the gel should be between 50 and 100 ng, which can be determined by comparing the intensity of the patient sample PCR products with that of

the DNA ladder. If the patient sample PCR products contain less than 50 to 100 ng, the PCR reaction should be repeated until sufficient quantity is obtained.

DNA Sequencing

For DNA sequencing, double stranded PCR products are labeled with four different fluorescent dyes, one specific for each nucleotide, in a cycle sequencing reaction. With Dye Terminator Chemistry, when one of these nucleotides is incorporated into the elongating sequence it causes a termination at that point. Over the course of the cycle sequencing reaction, the dye-labeled nucleotides are incorporated along the length of the PCR product generating many different length fragments.

The dye-labeled PCR products will separate according to size when electrophoresed through a polyacrylamide gel. At the lower portion of the gel on an ABI automated sequencer, the fragments pass through a region where a laser beam continuously scans across the gel. The laser excites the fluorescent dyes attached to the fragments causing the emission of light at a specific wavelength for each dye. Either a photomultiplier tube (PMT) detects the fluorescent light and converts is into an electrical signal (ABI 373) or the light is collected and separated according to wavelength by a spectrograph onto a cooled, charge coupled device (CCD) camera (ABI 377). In either case the data collection software will collect the signals and store them for subsequent sequence analysis.

PCR products are first purified for sequencing using a QIAQUICK-SPIN PCR PURIFICATION KIT (QIAGEN #28104). The purified PCR products are labeled by adding primers, fluorescently tagged dNTPs and Taq Polymerase FS in an ABI Prism Dye Terminator Cycle Sequencing Kit (PERKIN ELMER/ABI catalog #02154) in a PERKIN ELMER GENEAMP 9600 thermocycler.

The amounts of each component are:

For Samples	For Controls

Reagent	Volume	Reagent	Volume
Dye mix	8.0 μL	PGEM	2.0 μL
Primer (1.6 mM)	2.0 μL	M13	2.0 μL
PCR product	2.0 μL	Dye mix	8.0 μL
sdH2O	8.0 μL	sdH2O	8.0 μL

The thermocycling conditions are:

Temperature	Time	# of Cycles
96°C	15 sec. \	
50°C	5 sec. }	25
60°C	4 min. /	
4°C	hold	1

The product is then loaded into a gel and placed into an ABI DNA Sequencer (Models 373A & 377) and run. The sequence obtained is analyzed by comparison to the wild type (reference) sequence using SEQUENCE NAVIGATOR software. When a sequence does not align, it indicates a possible mutation. The DNA sequence is determined in both the forward and reverse directions. All results are provided to a second reader for review.

Heterozygous/homozygous point mutations and polymorphisms must be seen in both strands. Frame shift mutations will be seen in both strands and must have clear double peaks in frame shift regions to be so identified.

PCR Amplification for ASO

The genomic DNA is used as a template to amplify a separate DNA fragment encompassing the site of the mutation to be tested. The 50 ml PCR reaction contains the following components: 1 ml template (100 ng/ ml) DNA, 5.0 ml 10X PCR Buffer (PERKIN-ELMER), 2.5 ml dNTP (2mM each dATP, dCTP, dGTP, dTTP), 2.5 ml Forward Primer (10 mM), 2.5 ml Reverse Primer (10 mM), 0.5 ml (2.5 U total) AMPLITAQ7 TAQ DNA POLYMERASE or AMPLITAQ GOLD™ DNA POLYMERASE (PERKIN-ELMER), 1.0 to 5.0

ml (25 mM) $MgCl_2$ (depending on the primer) and distilled water (dH_2O) up to 50 ml. All reagents for each exon except the genomic DNA can be combined in a master mix and aliquoted into the reaction tubes as a pooled mixture.

For each exon analyzed, the following control PCRs are set up:

(1) "Negative" DNA control (100 ng placental DNA (SIGMA CHEMICAL CO., St. Louis, MO)

(2) Three "no template" controls

PCR for all exons is performed using the following thermocycling conditions:

Temperature	Time	Number of Cycles
95°C	5 min.(AMPLITAQ)	1
	or 10 min. (GOLD)	
95°C	30 sec. \	
55°C	30 sec. }	30 cycles
72°C	1 min /	
72°C	5 min.	1
4°C	hold	1

The quality control agarose gel of PCR amplification is performed as above.

Binding PCR Products to Nylon Membrane

The PCR products are denatured no more than 30 minutes prior to binding the PCR products to the nylon membrane. To denature the PCR products, the remaining PCR reaction (45 ml) and the appropriate positive control mutant gene amplification product are diluted to 200 ml final volume with PCR Diluent Solution (500 mM NaOH, 2.0 M NaCl, 25 mM EDTA) and mixed thoroughly. The mixture is heated to 95°C for 5 minutes, and immediately placed on ice and held on ice until loaded onto dot blotter, as described below.

The PCR products are bound to 9 cm by 13 cm nylon ZETA PROBE BLOTTING MEMBRANE (BIO-RAD, Hercules, CA, catalog number 162-0153) using a BIO-RAD dot

blotter apparatus. Forceps and gloves are used at all times throughout the ASO analysis to manipulate the membrane, with care taken never to touch the surface of the membrane with bare hands or latex gloves.

Pieces of 3MM filter paper [WHATMAN7, Clifton, NJ] and nylon membrane are pre-wet in 10X SSC prepared fresh from 20X SSC buffer stock. The vacuum apparatus is rinsed thoroughly with dH_2O prior to assembly with the membrane. 100 ml of each denatured PCR product is added to the wells of the blotting apparatus. Each row of the blotting apparatus contains a set of reactions for a single exon to be tested, including a placental DNA (negative) control, a synthetic oligonucleotide with the desired mutation or a PCR product from a known mutant sample (positive control), and three no template DNA controls.

After applying PCR products, the nylon filter is placed DNA side up on a piece of 3MM filter paper saturated with denaturing solution (1.5 M NaCl, 0.5 M NaOH) for 5 minutes. The membrane is transferred to a piece of 3MM filter paper saturated with neutralizing solution (1 M Tris-HCl, pH 8, 1.5 M NaCl) for 5 minutes. The neutralized membrane is then transferred to a dry 3MM filter DNA side up, and exposed to ultraviolet light (STRALINKER, STRATAGENE, La Jolla, CA) for exactly 45 seconds the fix the DNA to the membrane. This UV crosslinking should be performed within 30 min. of the denaturation/neutralization steps. The nylon membrane is then cut into strips such that each strip contains a single row of blots of one set of reactions for a single exon.

Hybridizing Labeled Oligonucleotides to the Nylon Membrane

Prehybridization

The strip is prehybridized at 52°C incubation using the HYBAID7 (SAVANT INSTRUMENTS, INC., Holbrook, NY) hybridization oven. 2X SSC (15 to 20 ml) is preheated to 52°C in a water bath. For each nylon strip, a single piece of nylon mesh cut slightly larger than the nylon membrane strip (approximately 1" x 5") is pre-wet with 2X SSC. Each single

nylon membrane is removed from the prehybridization solution and placed on top of the nylon mesh. The membrane/mesh "sandwich" is then transferred onto a piece of Parafilm™. The membrane/mesh sandwich is rolled lengthwise and placed into an appropriate HYBAID7 bottle, such that the rotary action of the HYBAID7 apparatus caused the membrane to unroll. The bottle is capped and gently rolled to cause the membrane/mesh to unroll and to evenly distribute the 2X SSC, making sure that no air bubbles formed between the membrane and mesh or between the mesh and the side of the bottle. The 2X SSC is discarded and replaced with 5 ml TMAC Hybridization Solution, which contains 3 M TMAC (tetramethyl ammoniumchloride - SIGMA T-3411), 100 mM Na_3PO_4(pH 6.8), 1 mM EDTA, 5X Denhardt's (1% Ficoll, 1% polyvinylpyrrolidone, 1% BSA (fraction V)), 0.6% SDS, and 100 mg/ml Herring Sperm DNA. The filter strips are prehybridized at 52°C with medium rotation (approx. 8.5 setting on the HYBAID7 speed control) for at least one hour. Prehybridization can also be performed overnight.

Labeling Oligonucleotides

The DNA sequences of the numerous oligonucleotide probes are used to detect the BRCA1 mutation. For each mutation, a mutant and a normal oligonucleotide must be labeled. While only five pairs of oligonucleotide probes are listed below, corresponding oligonucleotides for each mutation may be prepared and used in a similar manner.

mutation 185delAG.

wild-type 5'-AAT CTT AGA GTG TCC CA-3', SEQ ID NO:3

mutant 5'-ATC TTA GTG TCC CAC CT-3', SEQ ID NO:4

mutation 1136insA.

wild-type 5'-CAG AAA AAA AGG TAG AT-3', SEQ ID NO:5

mutant 5'-CAG AAA AAA AAG GTA GA-3', SEQ ID NO:6

mutation 5383insC.

wild-type 5'-AGA GAA TCC CAG GAC AG-3', SEQ ID NO:7

mutant 5'-AGA GAA TCC CCA GGA CA-3', SEQ ID NO:8

mutationC4446T.

wild-type 5'-AGG ACC TGC GAA ATC CA-3', SEQ ID NO:9

mutant 5'-AGG ACC TGT GAA ATC CA-3', SEQ ID NO:10

Each labeling reaction contains 2 ml 5X Kinase buffer (or 1 ml of 10X Kinase buffer), 5 ml gamma-ATP ^{32}P (not more than one week old), 1 µl T4 polynucleotide kinase, 3 µl oligonucleotide (20 mM stock), sterile H_2O to 10 µl final volume if necessary. The reactions are incubated at 37°C for 30 minutes, then at 65°C for 10 minutes to heat inactivate the kinase. The kinase reaction is diluted with an equal volume (10 µl) of sterile dH_20 (distilled water).

The oligonucleotides are purified on STE MICRO SELECT-D, G-25 spin columns (catalog no. 5303-356769), according to the manufacturer's instructions. The 20 µl synthetic oligonucleotide eluate is diluted with 80 µl dH_20 (final volume = 100 µl). The amount of radioactivity in the oligonucleotide sample is determined by measuring the radioactive counts per minute (cpm). The total radioactivity must be at least 2 million cpm. For any samples containing less than 2 million cpm total, the labeling reaction is repeated.

Hybridization with Mutant Oligonucleotides

Approximately 2-5 million cpm of the labeled mutant oligonucleotide probe is diluted into 5 ml of TMAC hybridization solution, containing 40 µl of 20 mM stock of unlabeled normal oligonucleotide. The probe mix is preheated to 52°C in the hybridization oven. The pre-hybridization solution is removed from each bottle and replaced with the probe mix. The filter is hybridized for 1 hour at 52°C with moderate agitation. Following hybridization, the probe mix is decanted into a storage tube and stored at -20°C. The filter is rinsed by adding approximately 20 ml of 2x SSC + 0.1% SDS at room temperature and rolling the capped bottle gently for

approximately 30 seconds and pouring off the rinse. The filter is then washed with 2x SSC + 0.1% SDS at room temperature for 20 to 30 minutes, with shaking.

The membrane is removed from the wash and placed on a dry piece of 3MM WHATMAN filter paper then wrapped in one layer of plastic wrap, placed on the autoradiography film, and exposed for about five hours depending upon a survey meter indicating the level of radioactivity. The film is developed in an automatic film processor.

Control Hybridization with Normal Oligonucleotides

The purpose of this step is to ensure that the PCR products are transferred efficiently to the nylon membrane.

Following hybridization with the mutant oligonucleotide, as described in the Examples above, each nylon membrane is washed in 2X SSC, 0.1% SDS for 20 minutes at 65°C to melt off the mutant oligonucleotide probes. The nylon strips are then prehybridized together in 40 ml of TMAC hybridization solution for at least 1 hour at 52°C in a shaking water bath. 2-5 million counts of each of the normal labeled oligonucleotide probes plus 40 µl of 20 mM stock of unlabeled normal oligonucleotide are added directly to the container containing the nylon membranes and the prehybridization solution. The filter and probes are hybridized at 52°C with shaking for at least 1 hour. Hybridization can be performed overnight, if necessary. The hybridization solution is poured off, and the nylon membrane is rinsed in 2X SSC, 0.1% SDS for 1 minute with gentle swirling by hand. The rinse is poured off and the membrane is washed in 2X SSC, 0.1% SDS at room temperature for 20 minutes with shaking.

The nylon membrane is removed placed on a dry piece of 3MM WHATMAN filter paper. The nylon membrane is then wrapped in one layer of plastic wrap and placed on autoradiography film, and exposure is for at least 1 hour.

For each sample, adequate transfer to the membrane is indicated by a strong autoradiographic hybridization signal. For each sample, an absent or weak signal when

hybridized with its normal oligonucleotide, indicates an unsuccessful transfer of PCR product, and it is a false negative. The ASO analysis must be repeated for any sample that did not successfully transfer to the nylon membrane.

Interpreting Results

After hybridizing with mutant oligonucleotides, the results for each exon are interpreted as follows:

Table 4A

Result	Interpretation	Action
 (+) (-) NT NT NT	All quality controls indicate assay is successful	Record results, dark circles are mutation positive, and all others are negative
(+) (-) NT NT NT	Assay not specific, mutant oligonucleotide hybridizing to normal DNA.	Rewash membrane 30 minutes longer at appropriate temperature and re-expose.
	Mutant oligonucleotide probe is either washed off or did not label well enough, or PCR product is not transferred to membrane efficiently.	Rehybridize with remaining labeled oligonucleotide. If still no signal, perform normal oligonucleotide hyb. as per the Examples to test transfer of PCR to membrane.

(+)	(-)		NT	NT	NT	
(+)	(-)		NT	NT	NT	Positive and negative controls indicate assay is successful, but PCR is contaminated.	Perform standard clean up procedures for PCR contamination.

After hybridization with normal oligonucleotides, interpret the results as follows:

Table 4B

(+)	(-)		NT	NT	NT	Results indicate transfer of PCR products to membrane is successful.	Record results.
(+)	(-)	#1	NT	NT	NT	Results indicate transfer of patient sample #1 is inefficient. May get false negative from this sample.	This sample will have to be transferred to another membrane and the assay repeated.

The sample #1 should be lighter than the controls. Patient samples containing a mutation are generally heterozygous and will hybridize to both the normal and mutant oligonucleotide probes.

DATA ON SPECIFIC MUTATIONS

The following specific mutations are examples of those which are presumed to be clinically significant for typing current cancer cells or a germ line mutation increasing the susceptibility to a tumor. A few of these mutations were also found by others as stated in TABLE 1 above.

TABLE 8
List of Nonsense Mutations

T127A, T127g, G144T, G147T, C153T, C174T, A177T, T184A, T184g, G186T, T191A, T200A, G204T, T208A, A213T, G216T, A231T, T236A, C251A, A252T, C260A, A267T, C279T, A282T, A285T, C295A, C295g, C297T, T302A, T307A, T307g, T311A, A312T, A327T, C339T, G342T, A351T, C360T, G369T, G372T, T379A, A381T, T392A, C399T, T415A, G417T, T422A, T422G, T434A, T434G, A444T, A447T, G450T, G465T, A474T, G480T, C495T, C509A, C509G, A510T, A522T, A525T, C534T, G540T, G546T, T559A, C561T, G564T, C582T, G597T, A606T, A621T, C624T, C633T, C639T, A642T, C656A, C656G, G660T, T664A, G666T, G681T, A696T, T707A, T707G, C710A, G717T, C723T, G726T, T730A, T733A, T733g, C735T, C747T, G750T, G762T, T772A, A783T, A786T, T797A, G798T, G807T, G828T, C837T, T856A, G867T, A870T, G882T, G894T, A897T, T902A, T902G, C903T, C919A, C919g, T925A, G933T, T941A, C964A, C964g, T967A, T967g, C969T, G975T, T988A, T988g, T991A, T991g, A999T, A1005T, G1017T, A1020T, G1026T, T1034A, A1038T, A1044T, C1047T, T1057A, T1057g, C1068T, A1077T, G1081A, G1082A, G1086T, A1092T, G1095T, T1103A, G1128T, A1131T, A1134T, T1163A, G1164T, A1167T, A1170T, G1173T, G1177A, G1178A, A1182T, C1185T, A1188T, C1199A, C1201A, C1201g, G1203T, A1212T, G1221T, G1234A, G1235A, C1257T, A1260T, G1269T, G1273A, G1274A, A1281T, G1290T, T1297A, T1297g, C1312A, C1312g, G1323T, G1329T, C1333A, C1333g, A1341T, T1357A, G1371T, G1380T, T1385A, T1385G, C1396A, C1396g, G1398T, A1401T, T1411A, T1411g, G1431T, T1438A, T1438g, T1445A, A1446T, G1452T, A1455T, A1467T, C1471A, C1471g, G1476T, G1488T, A1494T, A1506T, T1514A, T1514G, A1518T, A1521T, T1540A, T1540g, G1554T, G1569T, G1584T, C1590T, C1599T, G1602T, A1620T,

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TABLE 10
List of Two Base Deletions

4delGA, 5delAT, 6delTT, 7delTT, 8delTA, 9delAT, 10delTC, 11delCT, 12delTG, 132delGC, 133delCT, 134delTC, 135delCT, 136delTT, 137delTC, 138delCG, 139delGC, 140delCG, 141delGT, 142delTT, 143delTG, 144delGA, 145delAA, 146delAG, 147delGA, 148delAA, 149delAG, 150delGT, 151delTA, 152delAC, 153delCA, 154delAA, 155delAA, 156delAA, 157delAT, 158delTG, 159delGT, 160delTC, 161delCA, 162delAT, 163delTT, 164delTA, 165delAA, 166delAT, 167delTG, 168delGC, 169delCT, 170delTA, 171delAT, 172delTG, 173delGC, 174delCA, 175delAG, 176delGA, 177delAA, 178delAA, 179delAA, 180delAT, 181delTC, 182delCT, 183delTT, 184delTA, 185delAG, 186delGA, 187delAG, 188delGT, 189delTG, 190delGT, 191delTC, 192delCC, 193delCC, 194delCA, 195delAT, 196delTC, 197delCT, 198delTG, 199delGT, 200delTC, 201delCT, 202delTG, 203delGG, 204delGA, 205delAG, 206delGT, 207delTT, 208delTG, 209delGA, 210delAT, 211delTC, 212delCA, 213delAA, 214delAG, 215delGG, 216delGA, 217delAA, 218delAC, 219delCC, 220delCT, 221delTG, 222delGT, 223delTC, 224delCT, 225delTC, 226delCC, 227delCA, 228delAC, 229delCA, 230delAA, 231delAA, 232delAG, 233delGT, 234delTG, 235delGT, 236delTG, 237delGA, 238delAC, 239delCC, 240delCA, 241delAC, 242delCA, 243delAT, 244delTA, 245delAT, 246delTT, 247delTT, 248delTT, 249delTG, 250delGC, 251delCA, 252delAA, 253delAA, 254delAT, 255delTT, 256delTT, 257delTT, 258delTG, 259delGC, 260delCA, 261delAT, 262delTG, 263delGC, 264delCT, 265delTG, 266delGA, 267delAA, 268delAA, 269delAC, 270delCT, 271delTT, 272delTC, 273delCT, 274delTC, 275delCA, 276delAA, 277delAC, 278delCC, 279delCA, 280delAG, 281delGA, 282delAA, 283delAG, 284delGA, 285delAA, 286delAA, 287delAG, 288delGG, 289delGG, 290delGC, 291delCC, 292delCT, 293delTT, 294delTC, 295delCA, 296delAC, 297delCA, 298delAG, 299delGT, 300delTG, 301delGT, 302delTC, 303delCC, 304delCT, 305delTT, 306delTT, 307delTA, 308delAT, 309delTG, 310delGT, 311delTA, 312delAA, 313delAG, 314delGA, 315delAA, 316delAT, 317delTG, 318delGA, 319delAT, 320delTA, 321delAT, 322delTA, 323delAA, 324delAC, 325delCC, 326delCA, 327delAA, 328delAA, 329delAA, 330delAG, 331delGG, 332delGA, 333delAG, 334delGC, 335delCC, 336delCT, 337delTA, 338delAC, 339delCA, 340delAA, 341delAG, 342delGA, 343delAA, 344delAA, 345delAG, 346delGT, 347delTA, 348delAC, 349delCG, 350delGA, 351delAG, 352delGA, 353delAT, 354delTT, 355delTT, 356delTA, 357delAG, 358delGT, 359delTC, 360delCA, 361delAA, 362delAC, 363delCT, 364delTT, 365delTG, 366delGT, 367delTT, 368delTG, 369delGA, 370delAA, 371delAG, 372delGA, 373delAG, 374delGC, 375delCT, 376delTA, 377delAT, 378delTT, 379delTG, 380delGA, 381delAA, 382delAA, 383delAA, 384delAT, 385delTC, 386delCA, 387delAT, 388delTT, 389delTT, 390delTG, 391delGT, 392delTG, 393delGC, 394delCT, 395delTT, 396delTT, 397delTT, 398delTC, 399delCA, 400delAG, 401delGC, 402delCT, 403delTT, 404delTG, 405delGA, 406delAC, 407delCA, 408delAC, 409delCA, 410delAG, 411delGG, 412delGT,

413delTT, 414delTT, 415delTG, 416delGG, 417delGA, 418delAG, 419delGT, 420delTA, 421delAT, 422delTG, 423delGC, 424delCA, 425delAA, 426delAA, 427delAC, 428delCA, 429delAG, 430delGC, 431delCT, 432delTA, 433delAT, 434delTA, 435delAA, 436delAT, 437delTT, 438delTT, 439delTT, 440delTG, 441delGC, 442delCA, 443delAA, 444delAA, 445delAA, 446delAA, 447delAA, 448delAG, 449delGG, 450delGA, 451delAA, 452delAA, 453delAA, 454delAT, 455delTA, 456delAA, 457delAC, 458delCT, 459delTC, 460delCT, 461delTC, 462delCC, 463delCT, 464delTG, 465delGA, 466delAA, 467delAC, 468delCA, 469delAT, 470delTC, 471delCT, 472delTA, 473delAA, 474delAA, 475delAA, 476delAG, 477delGA, 478delAT, 479delTG, 480delGA, 481delAA, 482delAG, 483delGT, 484delTT, 485delTT, 486delTC, 487delCT, 488delTA, 489delAT, 490delTC, 491delCA, 492delAT, 493delTC, 494delCC, 495delCA, 496delAA, 497delAA, 498delAG, 499delGT, 500delTA, 501delAT, 502delTG, 503delGG, 504delGG, 505delGC, 506delCT, 507delTA, 508delAC, 509delCA, 510delAG, 511delGA, 512delAA, 513delAA, 514delAC, 515delCC, 516delCG, 517delGT, 518delTG, 519delGC, 520delCC, 521delCA, 522delAA, 523delAA, 524delAA, 525delAG, 526delGA, 527delAC, 528delCT, 529delTT, 530delTC, 531delCT, 532delTA, 533delAC, 534delCA, 535delAG, 536delGA, 537delAG, 538delGT, 539delTG, 540delGA, 541delAA, 542delAC, 543delCC, 544delCC, 545delCG, 546delGA, 547delAA, 548delAA, 549delAA, 550delAT, 551delTC, 552delCC, 553delCT, 554delTT, 555delTC, 556delCC, 557delCT, 558delTT, 559delTG, 560delGC, 561delCA, 562delAG, 563delGG, 564delGA, 565delAA, 566delAA, 567delAC, 568delCC, 569delCA, 570delAG, 571delGT, 572delTC, 573delCT, 574delTC, 575delCA, 576delAG, 577delGT, 578delTG, 579delGT, 580delTC, 581delCC, 582delCA, 583delAA, 584delAC, 585delCT, 586delTC, 587delCT, 588delTC, 589delCT, 590delTA, 591delAA, 592delAC, 593delCC, 594delCT, 595delTT, 596delTG, 597delGG, 598delGA, 599delAA, 600delAC, 601delCT, 602delTG, 603delGT, 604delTG, 605delGA, 606delAG, 607delGA, 608delAA, 609delAC, 610delCT, 611delTC, 612delCT, 613delTG, 614delGA, 615delAG, 616delGG, 617delGA, 618delAC, 619delCA, 620delAA, 621delAA, 622delAG, 623delGC, 624delCA, 625delAG, 626delGC, 627delCG, 628delGG, 629delGA, 630delAT, 631delTA, 632delAC, 633delCA, 634delAA, 635delAC, 636delCC, 637delCT, 638delTC, 639delCA, 640delAA, 641delAA, 642delAA, 643delAG, 644delGA, 645delAC, 646delCG, 647delGT, 648delTC, 649delCT, 650delTG, 651delGT, 652delTC, 653delCT, 654delTA, 655delAC, 656delCA, 657delAT, 658delTT, 659delTG, 660delGA, 661delAA, 662delAT, 663delTT, 664delTG, 665delGG, 666delGG, 667delGA, 668delAT, 669delTC, 670delCT, 671delTG, 672delGA, 673delAT, 674delTT, 675delTC, 676delCT, 677delTT, 678delTC, 679delCT, 680delTG, 681delGA, 682delAA, 683delAG, 684delGA, 685delAT, 686delTA, 687delAC, 688delCC, 689delCG, 690delGT, 691delTT, 692delTA, 693delAA, 694delAT, 695delTA, 696delAA, 697delAG, 698delGG, 699delGC, 700delCA, 701delAA, 702delAC, 703delCT, 704delTT, 705delTA, 706delAT, 707delTT, 708delTG, 709delGC, 710delCA, 711delAG, 712delGT, 713delTG, 714delGT, 715delTG, 716delGG, 717delGG, 718delGA, 719delAG, 720delGA, 721delAT, 722delTC, 723delCA, 724delAA,

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1095delGA, 1096delAA, 1097delAA, 1098delAC, 1099delCA, 1100delAT, 1101delTG,
1102delGT, 1103delTA, 1104delAA, 1105delAT, 1106delTG, 1107delGA, 1108delAT,
1109delTA, 1110delAG, 1111delGG, 1112delGC, 1113delCG, 1114delGG, 1115delGA,
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1403delAA, 1404delAT, 1405delTA, 1406delAG, 1407delGA, 1408delAC, 1409delCT,
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TABLE 11
List of One Base Insertions
(N= A, T, G, or C)

122insN, 123insN, 124insN, 125insN, 126insN, 127insN, 128insN, 129insN, 130insN, 131insN, 132insN, 133insN, 134insN, 135insN, 136insN, 137insN, 138insN, 139insN, 140insN, 141insN, 142insN, 143insN, 144insN, 145insN, 146insN, 147insN, 148insN, 149insN, 150insN, 151insN, 152insN, 153insN, 154insN, 155insN, 156insN, 157insN, 158insN, 159insN, 160insN, 161insN,

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TABLE 12
List of Two Base Insertions
(NN= AA, AT, AG, AC, TA, TT, TG, TC, GA, GT, GG, GC, CA, CT, CG, or CC)

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The present invention is not to be limited in scope by the specific embodiments described herein, which are intended as single illustrations of individual aspects of the invention, and functionally equivalent methods and components are within the scope of the invention. Indeed,

various modifications of the invention, in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the appended claims.

All references mentioned herein are incorporated by reference.